Comparing direct and indirect selfing rate estimates: When are population-structure estimates reliable?

Anja Bürkli[1,2,*] , Natalie Sieber[1,2] , Katri Seppälä[1] & Jukka Jokela[1,2]

[1]EAWAG, Swiss Federal Institute of Aquatic Science and Technology, Department of Aquatic Ecology, 8600 Dübendorf, Switzerland.

[2]ETH Zurich, D-USYS, Institute of Integrative Biology, 8092 Zürich, Switzerland.

* Corresponding author

Anja Bürkli: anja.buerkli@eawag.ch, +41 (0)58 765 67 32

Jukka Jokela: jukka.jokela@eawag.ch, +41 (0)58 765 51 71

Running title: Direct and indirect selfing rate estimates

Word count: 6210

Abstract

The rate of self-fertilization (i.e. selfing) is a key evolutionary parameter in hermaphroditic species, yet obtaining accurate estimates of selfing rates in natural populations can be technically challenging. Most published estimates are derived from population-level heterozygote deficiency (i.e. F_{IS}) or identity disequilibria (e.g. the software RMES). These indirect methods can be applied to population genetic survey data, while direct methods using progeny arrays require much larger datasets that are often difficult to collect in natural populations or even require captive breeding. Unfortunately, indirect methods rely on assumptions that can be problematic, such as negating biparental inbreeding, inbreeding disequilibrium, and (for F_{IS}) the presence of null alleles. The performance of indirect estimates against progeny-array estimates is still largely unknown. Here we used both direct progeny-array and indirect population-level methods to estimate the selfing rate in a single natural population of the simultaneously hermaphroditic freshwater snail *Radix balthica* throughout its reproductive lifespan using ten highly polymorphic microsatellites. We found that even though progeny arrays (n=1034 field-collected embryos from 60 families) did not reveal a single selfed embryo, F_{IS}-based selfing rates (n=316 adults) were significantly positive in all six

sequential population samples. Including a locus with a high frequency of null alleles further biased F_{IS}-based estimates. Conversely, RMES-based estimates were very similar to progeny-array estimates and proved insensitive to null alleles. The assumptions made by RMES were thus either met or irrelevant in this particular population, making RMES a valid, cost-efficient alternative to progeny arrays.

Keywords

identity disequilibrium, inbreeding coefficient, mating system evolution, null alleles, progeny arrays, *Radix balthica*

Introduction

The rate of self-fertilization (i.e. selfing) affects the population genetic and evolutionary properties of hermaphroditic species in various ways. Frequent selfing decreases heterozygosity within individuals, reduces effective population size, can lead to inbreeding depression and may ultimately impair the adaptive potential of a population (Charlesworth and Charlesworth, 1987). At the same time, selfing provides fertilization assurance, increases the transmission of genes, releases most species of costs of mating and mate acquisition, and allows successful multilocus genotypes to be passed on with relatively few alterations (Fisher, 1941; Baker, 1955; Allard, 1975; Jarne and Charlesworth, 1993; Avise and Mank, 2009).

Despite the profound impact of selfing on a species, we still know relatively little about the distribution of selfing rates among hermaphroditic animals. In a survey of 142 animal species, 47% had intermediate selfing rates, ~36% were predominant outcrossers and ~17% predominant selfers (Jarne and Auld, 2006). However, 97% of these estimates were based on population-level heterozygote deficiency (i.e. the inbreeding coefficient F_{IS}), a method that has some rather strict assumptions. For example, inbreeding coefficient-based selfing rate estimates assume that there is no biparental inbreeding, that the population is

not subdivided, that inbreeding is homogeneous across generations, that allele and genotype frequencies are at equilibrium, and that there are no null alleles, genotyping errors or new mutations biasing the analysis. When estimating the "primary" selfing rate at birth or oviposition, as opposed to a selfing rate measured in juveniles or adults (Ritland, 1990), one also needs to assume that pre-sampling mortality has not changed the proportion of selfed to outcrossed individuals in a population (David *et al.*, 2007; Jarne and David, 2008; Wang *et al.*, 2012).

Not all these assumptions may be equally severe, but understandably empirical studies usually cannot produce powerful tests for all of them, or evaluate in detail which assumptions are more critical than others. Unfortunately, we can expect that violations of these assumptions often result in inflated selfing rates, rendering inbreeding coefficient-based estimates prone to error (David *et al.*, 2007; Jarne and David, 2008; Escobar *et al.*, 2011). Fortunately, a method has been developed that uses correlations of heterozygosity among loci (i.e. identity disequilibria) to estimate selfing rates (David *et al.*, 2007). The method has been implemented in a software package named "robust multilocus estimate of selfing" (RMES) and promises a significant improvement of population-level estimates because its estimates are thought to be immune to the distorting effect of null alleles. However, also RMES-based selfing rate estimates do share some

of the assumptions of F_{IS}-based estimates, for example that biparental inbreeding and, when estimating "primary" selfing rates, in- or outbreeding depression prior to sampling can be ignored, and that the population is in inbreeding equilibrium (Wang *et al.*, 2012). In comparisons where both methods were used on the same molecular data many species classified as outcrossers by RMES were classified as having intermediate selfing rates (i.e. as being mixed-maters) by the inbreeding coefficient (David *et al.*, 2007; Escobar *et al.*, 2011). In contrast, the bias introduced by null alleles into F_{IS}-based estimates was less noticeable in highly selfing species, in which null alleles often occur as easily detectable null homozygotes (David *et al.*, 2007; Escobar *et al.*, 2011).

Alternatively, selfing rates can be estimated directly using progeny arrays (Ritland and Jain, 1981). In progeny-array analysis, selfing events are identified directly by comparing a mother's genotype to the genotypes of her offspring. Although substantially more cost- and labor-intensive, selfing rates based on progeny arrays are almost free of problematic assumptions, especially when computed using progenies collected in the field (to ensure fertilization events are free of laboratory artifacts) and, if "primary" selfing rates are to be estimated, when progenies are genotyped at a very early stage in life (to minimize bias due to pre-sampling mortality caused by in- or outbreeding

depression) (Jarne and David, 2008; Wang *et al.*, 2012). Progeny-array methods also offer the possibility to examine variation in selfing rates among individuals (Jarne and David, 2008; Wang *et al.*, 2012), as well as the certainty that all identified selfing events must have occurred in the last generation. By contrast, population-level estimates assess the frequency of selfing and biparental inbreeding over several generations (David *et al.*, 2007; Jarne and David, 2008; Wang *et al.*, 2012). To yield reliable estimates, progeny-array methods require the use of genetic markers with sufficient resolution to unequivocally identify outcrossed and selfed individuals (Bernatchez and Duchesne, 2000; Jarne and David, 2008). This is particularly important when genotyping field-collected progenies with unknown mothers, which makes it much harder to infer parenthood than when mothers can be genotyped along with their offspring, as is usually the case in laboratory studies. Allozyme polymorphism, used for estimating 97% of the selfing rates compiled by Jarne & Auld (2006), unfortunately often fails to provide the required resolution, and the same is true for small sets of weakly polymorphic microsatellite loci.

Presuming that technical problems such as null alleles are absent and that statistical power is adequate, we therefore expect the comparison of progeny-array and population-level estimates to provide complementary insights into the history of a studied mating system. Nevertheless, for the most

part we do not know how often indirect population-level estimates deviate from direct progeny-array estimates, be that for technical or biological reasons, as both types of methods have very rarely been applied simultaneously to the same population with sufficient statistical power. In 14 studies that estimated both F_{IS}- and progeny-array-based selfing rates in the same populations of hermaphroditic animals, the mean absolute difference (± SD) between both types of estimates was 0.11 ± 0.13 (Table 1, full references provided in the Supplementary Material). In seven studies F_{IS}-based and in five studies progeny-array-based estimates were higher. In two studies both estimates were identical, one of them analyzing a purely outcrossing population and one two purely selfing populations. This finding tallies with the expectation that differences between methods should be smallest at the distribution's extremes. Only one study also estimated selfing rates using RMES, and found RMES- and F_{IS}-based estimates to be very similar and slightly lower than progeny-array estimates (Kupfernagel *et al.*, 2010). Although these results seem reassuring, it should be noted that almost all these studies exclusively used progenies produced in the laboratory, in most cases by adult individuals caught in the field and then kept in isolation. Apart from potentially altering natural patterns of fertilization, the isolation treatment assumes that mothers had obtained sufficient amounts of allosperm before being captured and can store it long

147 enough for successful allofertilization. If not met, these assumptions may lead
148 to artificially increased selfing rates in progenies, potentially masking the true
149 differences between methods.

150 As a consequence, our current knowledge about the distribution of selfing rates
151 among hermaphroditic animals, and especially about the proportion and identity
152 of mixed-maters, is still far from complete (Jarne and Auld, 2006; Jarne and
153 David, 2008; Escobar *et al.*, 2011). Biased selfing rate estimates will also result
154 in incorrect inferences drawn about the population genetic and evolutionary
155 properties of individual populations and species.

156 Here we estimated both "primary" selfing rates and selfing rates among adult
157 individuals in a natural population of the annual, simultaneously
158 hermaphroditic freshwater snail *Radix balthica*. We used progeny arrays, the
159 inbreeding coefficient and RMES, and covered the reproductive lifespan from
160 beginning to end. *R. balthica* has been suspected of being a mixed-mater
161 (Coutellec-Vreto *et al.*, 1997; Wiehn *et al.*, 2002; Jokela *et al.*, 2006;
162 Pfenninger *et al.*, 2011; Haun *et al.*, 2012; but see also Jarne and Delay, 1990),
163 but for reasons mentioned above these selfing rate estimates may come with
164 some uncontrolled bias. We used ten highly polymorphic microsatellite loci as
165 genetic markers and ensured that selfing rates were free of laboratory artifacts,
166 the influence of in- or outbreeding depression, errors caused by incomplete

sampling, and contamination with samples from other species. The comparison of indirect population-level estimates both affected and unaffected by null alleles with direct individual-level estimates based on progeny arrays enabled us to quantify the potential bias introduced by unmet assumptions and technical problems (i.e. null alleles) in the former two methods. In addition, we assessed the magnitude of bias caused by the presence of known null alleles in a single locus directly by running all analyses both with and without this locus. We found that selfing rates estimated using RMES were very similar to progeny-array estimates, making this method a valid, cost-efficient alternative, while inbreeding coefficient-based estimates suffered heavily from the presence of null alleles.

Materials and Methods

Study system

Radix balthica is a diploid, simultaneously hermaphroditic freshwater snail that lives in the shallow littoral zone of large water bodies throughout Europe from Iceland to Mediterranean Countries (Cordellier and Pfenninger, 2009; Pfenninger *et al.*, 2011; Lawton *et al.*, 2015). In Lake Zurich, Switzerland, *R. balthica* hatches from eggs in spring and reaches sexual maturity by the end of the year. The reproductive season starts in late February or early March and lasts until May. Adult snails die after they reproduce and by late May the adult cohort is completely replaced by juveniles. Thus, generations are non-overlapping and generation time is about one year. During the egg-laying period (March to May), snails may copulate repeatedly in both sexual roles and lay hundreds of eggs in distinct egg clutches (A. Bürkli unpublished data). The population studied here (Uerikon, Lake Zurich, approx. 4700 m^2 of suitable habitat) has been surveyed for parasitological studies, population genetic studies and teaching purposes occasionally since almost 20 years, during which population size has remained constant and fairly large (>10’000 individuals, J. Jokela unpublished data).

As the species is phenotypically very variable (e.g. Brönmark *et al.*, 2011; Schniebs *et al.*, 2011; Ahlgren *et al.*, 2013), the taxonomic identification of *Radix* snails is notoriously difficult (Pfenninger *et al.*, 2006; Schniebs *et al.*, 2011; Lawton *et al.*, 2015). We therefore verified that only individuals of *R. balthica* were included in this study by sequencing the mitochondrial cytochrome oxidase subunit I (COI) gene, shown to be well suited for phylogenetic species delineation in *Radix* (Lawton *et al.*, 2015), and by microsatellite genotyping (for details see Supplementary Methods 1).

Collection of egg clutches and adult snails

We collected adult snails and egg clutches from a relatively large area (approx. 900 m^2) using snorkelling equipment. Adult snails served as candidate parents in parentage analyses of progeny arrays and were used to estimate indirect population-level selfing rates at the level of adults, while egg clutches served for estimating "primary" selfing rates based on progeny arrays. Adult snails were caught at peak breeding season in three consecutive years (24.04.2013, 11.04.2014, 13.04.2015) to be able to test for potential temporal differences in estimated selfing rates. Adults were also caught on three additional dates in 2014, spanning the reproductive season from its very beginning (06.03.2014) to its very end (23.05.2014). Egg clutches were collected on the same four dates in 2014 on which adult snails were caught, resulting in four and six samples for

progeny-array and population-level analyses, respectively. Egg clutches were gently detached from boulders using a plastic spoon, and adult snails were collected by hand. All samples were brought to the laboratory in Eawag-Dübendorf, where adults were killed and preserved for genotyping at -80°C, either immersed in 70% ethanol or dry. Egg clutches were placed in individual, water-filled 40 cl plastic cups (room temperature 18°C) and allowed to develop until hatching was imminent, at which point they were transferred to individual 1.5 ml plastic tubes and stored at -80°C as well. Later, egg clutches were thawed and the number of developed and undeveloped embryos was counted in each clutch using a dissection microscope.

Genetic analysis

We estimated the selfing rate from 15 egg clutches and from 53 ± 20 (mean ± SD) adult snails per sampling day. We genotyped 15 embryos per clutch irrespective of clutch size, the only exception being small clutches with <15 embryos, of which all embryos were genotyped. When present, we also genotyped undeveloped embryos. This was done to evaluate the sampling bias caused by a potential over- or underrepresentation of selfed offspring among undeveloped embryos. However, undeveloped embryos were very rare (30/1034 (2.9%) genotyped embryos, and 105/3038 (3.5%) eggs present in the 60 clutches). Sample sizes of adult snails and embryos are listed in Tables 2 and

3, respectively. All samples were genotyped for ten microsatellite markers (GenBank Accession No. KX830983-KX830992) that had been newly developed for this population by Ecogenics (Zurich, Switzerland). We tried to minimize scoring errors by running an extensive pilot study and performing independent repeatability tests using newly extracted DNA on 17% of adult genotypes (Pompanon *et al.*, 2005). Details of markers and genotyping routines are provided in Table 4 and Supplementary Methods 2.

Screening for null alleles

We screened all loci for the presence of null alleles using 2044 multilocus genotypes of snails originating from the Uerikon population. This included all samples genotyped for this study, but we also used all additional material we had available. For each locus, we counted the number of genotypes that could not be scored, either because no microsatellite peak was present (i.e. "missing peaks"), more than two peaks were present, or peaks were ambiguous in other ways (e.g. lacking the locus-specific shape). Overall, 1392 / 20440 (6.8%) locus-specific genotypes could not be scored, and most of them (75.6%) were due to missing peaks. Missing peaks can result from amplification failure caused by mutations in the primer region, and may thus be indicative of null allele homozygotes (Dabrowski *et al.*, 2015). We therefore computed locus-specific frequencies of null alleles from the frequency of genotypes with

missing peaks (following Dabrowski *et al.*, 2015), and frequencies of other types of genotyping errors based on the frequency of genotypes that could not be scored for other reasons. All locus-specific error rates are listed in Table 4. In addition, we compared the genotypes of 59 maternal and 1320 juvenile snails reared in the laboratory to identify mother-offspring mismatches (data not shown).

Selfing rates based on progeny arrays

Parentage analyses were performed using COLONY version 2.0.5.9 (Jones and Wang, 2010). COLONY does not require previous knowledge of maternal genotypes but rather reconstructs them based on the offspring genotypes present in an egg clutch, making it possible to estimate selfing rates of field-collected clutches with unknown mothers. After reconstructing maternal genotypes, COLONY returns for each embryo the probability of being self-fertilized. This kind of progeny-array approach has the advantage of estimating the natural selfing rate at oviposition (i.e. the "primary" selfing rate in the population), but comes at the disadvantage of complicating the inference of parenthood. Parentage was inferred simultaneously for clutches collected on the same day. Adult genotypes from the same sampling day were entered into COLONY both as candidate mothers and fathers, and clutch identities were specified as maternal sibships. As parentage assignments can be distorted by

277 both null alleles (Dakin and Avise, 2004; Dabrowski *et al.*, 2015), known to be
278 common in molluscs (e.g. Kopp *et al.*, 2012), and genotyping errors, we
279 included the previously computed locus-specific error rates due to null alleles
280 and due to other types of genotyping errors (see above) in all parentage
281 analyses (Jones and Wang, 2010).

282 The selfing rate of an egg clutch will only be known without error if every
283 single embryo present in the clutch is genotyped. To estimate the error
284 introduced by genotyping only a subset of embryos we genotyped seven egg
285 clutches of different sizes (36.3 ± 16.3 eggs (mean ± SD)) as comprehensively
286 as possible (91.4 ± 5.2% of all eggs genotyped). Five of these clutches were
287 collected at peak and two at the end of the breeding season, when we expected
288 selfing rates to be lowest and the potential error caused by sampling
289 incompleteness to be highest.

290 Mean selfing rates and associated 95% confidence intervals for each of the four
291 sampling days were computed after pooling the probabilities of being selfed for
292 all embryos collected on the same sampling day – regardless of clutch
293 identities. However, means and confidence intervals that were computed from
294 the residuals of a linear model of probability of being selfed versus clutch
295 identity, thus correcting for potential differences among clutches, were very
296 similar. To make sure that statistical power among sampling days and egg

clutches was identical when computing means and confidence intervals, we restricted the dataset to 15 randomly chosen embryos in clutches where >15 embryos had been genotyped. Even so, including all genotyped embryos did not change estimates noticeably.

Selfing rates based on adult genotypes

We estimated the number of polymorphic loci, the mean number of alleles per locus, and the observed and expected heterozygosity, the latter calculated without bias (Nei, 1978), for each of the six groups of adults collected on the same day using Genetix, version 4.05.2 (Belkhir *et al.*, 1996-2004). The same software was used to compute population-level estimates of the inbreeding coefficient F_{IS} over all loci according to Weir and Cockerham (1984) with a 95% confidence interval based on 10'000 bootstrap iterations. The inbreeding coefficient-based population-level selfing rate ($s(F_{IS})$) was then obtained from the relationship $s=2F_{IS}/(1+F_{IS})$ (Wright, 1969). The 95% CI for $s(F_{IS})$ was computed based on the variance of $s(F_{IS})$ and served to assess the significance of $s(F_{IS})$. Var($s(F_{IS})$) was estimated using equation 2 in Jarne & David (2008), modified to accommodate multiple loci by substituting the single-locus sampling variance of F_{IS} with the multilocus sampling variance estimated in Genetix using the jackknife (Belkhir *et al.*, 1996-2004), and the single-locus F_{IS} with the multilocus F_{IS}.

In addition, we computed selfing rates that should be unaffected by the presence of null alleles using the software RMES (David *et al.*, 2007). RMES implements two methods for estimating selfing rates, one that uses two-locus heterozygosity disequilibrium values (g_2, with associated selfing rate $s(g_2)$) and one that maximizes the log-likelihood of the multilocus heterozygosity structure of a sample (s(ML)). As a significance test for $s(g_2)$ RMES computes the probability that there is no selfing (g_2=s=0), here obtained from 10'000 iterations of randomly reassorting single-locus heterozygosities among individuals. A 95% CI for $s(g_2)$ was obtained by computing a 95% CI for g_2 and transforming it back to the scale of s using equation 9 in David et al. (2007). s(ML) was calculated with a precision for the log-likelihood of 0.00001 and a maximum of 10 generations of selfing allowed for a heterozygote (kmax=10). A 95% CI for s(ML) was provided by RMES (David *et al.*, 2007) and was also used to judge the significance of s(ML).

Results

Presence of null alleles

Missing microsatellite peaks occurred in varying frequency at all loci, suggesting that all loci suffered from null alleles, but some loci more so than others. The frequency of null alleles ranged from 2.8% (locus Rb_8) to 8.2% (locus Rb_3) with an average of 5.1% (Table 4). Tellingly, locus Rb_3 also showed the highest frequency of ambiguous peaks (4.7%) and caused numerous mismatches between maternal and offspring genotypes in lab-reared snails (data not shown). We therefore computed all selfing rates twice, once without locus Rb_3 to obtain estimates that were largely unaffected by null alleles, and once including it to quantify the bias introduced by it.

Allelic diversity

The number of alleles observed per locus in the nine loci that were largely free from null alleles ranged from 7 to 29 with an average of 16.9 (Table 4). Within groups of 15 embryos, each sampled from a separate clutch on the same day, the mean number of alleles per locus (± SD) was 7.1 ± 3.2 (Table 4). The number of alleles was very similar within groups of 15 randomly chosen adults sampled on the same day (7.6 ± 3.0, Table 4). Such substantial allelic richness

was reflected in high values of observed single-locus heterozygosity, both among embryos (mean ± SD: 0.64 ± 0.27) and adults collected on the same day (0.66 ± 0.20, Table 4). Including locus Rb_3, which showed an increased frequency of null alleles, did not change these numbers substantially (Table 4). We also estimated the probability of identity, which is the probability that a multilocus genotype is shared by randomly drawn adult snails (P(ID)unbiased from Waits *et al.*, 2001). When including locus Rb_3, this probability is between 6.8×10^{-14} and 3.8×10^{-12} for all six sampling days. When excluding the locus, probabilities of identity are slightly higher, but still extremely low (between 1.8×10^{-11} and 2.7×10^{-10}). Statistical power in our dataset should thus be both adequate and sufficient to estimate selfing rates without any bias caused by a lack of allelic diversity.

No selfing in field-collected egg clutches

Among 1034 successfully genotyped embryos from a total of 60 egg clutches not a single embryo was more likely to be selfed than to be outcrossed (Table 3). Seven of these egg clutches were genotyped as comprehensively as possible so as not to overlook very rare selfing events, but still revealed no selfed embryos. As a consequence, mean selfing rates based on all embryos collected on a sampling day were zero or extremely close to zero on all four sampling days, regardless of the inclusion of locus Rb_3 (i.e. mean selfing rates 0.000-

0.003, Figure 1, Table 3, Supplementary Table 1). When excluding locus Rb_3, thereby restricting analyses to nine loci largely free from null alleles, just three embryos had slightly elevated individual probabilities of being selfed (i.e. probabilities 0.06, 0.26, 0.39), while all other embryos were selfed with probabilities of <0.01 (Table 3). The three deviant embryos all came from different clutches and were collected either at the begin or at the end of the reproductive season (Table 3). When including locus Rb_3, heavily affected by null alleles, the number of embryos with slightly elevated individual probabilities of being selfed increased to ten (i.e. probabilities 0.01-0.44, mean 0.11; Table 3). These probabilities are below the threshold of 0.5, indicating that all embryos were still more likely to be outcrossed than to be selfed. One out of 30 undeveloped embryos (3.3%) and, depending on the inclusion of locus Rb_3, two to nine out of 1004 developed embryos (0.2-0.9%) had an elevated probability to be selfed (Table 3). As the 95% confidence intervals of these proportions are overlapping, the difference between undeveloped and developed embryos is not statistically significant (0.0008-0.17 vs. 0.0002-0.007 or 0.004-0.017, respectively, Zar, 1996).

Apparent selfing in field-collected adults due to null-allele bias

Multilocus inbreeding coefficients (F_{IS}) across the nine loci that were largely free from null alleles were low (0.039 – 0.109, mean 0.071), but significantly positive on four of the six sampling days (Table 2). Accordingly, population-level selfing rates based on the inbreeding coefficient were relatively low yet significantly different from zero, ranging from 7.4% to 19.7% with an average of 13.1% (Figure 1, Supplementary Table 1). Including locus Rb_3 almost doubled F_{IS}-values (0.120-0.170, mean 0.133), rendering them significantly positive on all six sampling days (Table 2). As a consequence, mean $s(F_{IS})$ increased to 23.4% (21.5%-29.0%; Figure 1, Supplementary Table 1).

Selfing rates computed based on the excess of doubly heterozygous genotypes, a method not sensitive to the distorting effect of null alleles (David *et al.*, 2007), were considerably lower, only averaging 2.9% ($s(g_2)$) and 1.7% (s(ML)), respectively (Figure 1, Supplementary Table 1). Only one of the twelve date- and method-specific selfing rate estimates was marginally significant (s(ML) on sampling day 22.03.2014). The inclusion of locus Rb_3 did not change these estimates markedly (mean 2.0% ($s(g_2)$) and mean 1.3% (s(ML)), Figure 1, Supplementary Table 1), nor did it change their statistical significance.

There were no significant temporal differences in selfing rates when considering $s(g_2)$ and s(ML), independent of the inclusion of locus Rb_3, as confidence intervals on all sampling days were overlapping (Figure 1, Supplementary Table 1). The selfing rate based on the inbreeding coefficient, on the other hand, did show some significant variation over time. According to F_{IS}, selfing was most common at the beginning of 2014 (both with and without locus Rb_3), and least common in 2015 (only without locus Rb_3, Figure 1, Supplementary Table 1).

Comparison of selfing rates between methods

On all sampling days and irrespective of the inclusion of locus Rb_3, selfing rates based on progeny arrays were lowest and could be estimated with the highest precision (Figure 1, Supplementary Table 1). In contrast, inbreeding coefficient-based estimates significantly exceeded progeny-array estimates in all direct comparisons (Figure 1, Supplementary Table 1). Meanwhile, selfing rates based on a point estimate of the second-order heterozygosity disequilibrium (g_2), computed using the software RMES, were never significantly different from progeny-array estimates, as all confidence intervals of $s(g_2)$ included zero (Figure 1, Supplementary Table 1). Similarly, maximum-likelihood estimates of the selfing rate, also provided by RMES, overlapped with progeny-array estimates in 6/8 (75.0%) direct comparisons, the

sole exception being sampling day 22.03.2014 independent of the inclusion of locus Rb_3 (Figure 1, Supplementary Table 1).

Discussion

Our study shows that inbreeding coefficient-based selfing rate estimates can be significantly positive even though progeny-array analysis indicates complete outcrossing. We found that most of this upward bias seems to be caused by null alleles, highlighting the importance of a detailed technical assessment of marker performance as part of the study. Had we estimated selfing rates solely based on inbreeding coefficients, we would have erroneously concluded that our study population is moderately mixed-mating. We would have been more confident about this erroneous conclusion had we failed to exclude the single locus with a high frequency of null alleles. This finding demonstrates how severely null alleles can distort selfing rate estimates that rely on heterozygote deficiency (David *et al.*, 2007; Wang *et al.*, 2012), and how carefully one needs to examine the properties of the available molecular markers. Given how difficult it is to develop genetic markers that are both sufficiently powerful and entirely free of null alleles, it is probably safest to stop using the inbreeding coefficient in its current form for estimating selfing rates with this type of markers.

Our study also shows that selfing rate estimates obtained from the software RMES, which deploys identity disequilibria-based algorithms that are not sensitive to null alleles (David *et al.*, 2007; Wang *et al.*, 2012), effectively

corrected the shortcomings of the inbreeding coefficient-based method and yielded estimates very similar to those from progeny arrays. It appears that the assumptions made by RMES about the lack of biparental inbreeding, inbreeding disequilibrium and cryptic population subdivision were largely met in the particular population studied here. Apart from corroborating the general absence of selfing, the similarity of the two types of estimates provides additional insights into the studied mating system. First, it suggests that not only selfing but also biparental forms of inbreeding are rare, seeing that population-level estimates, which would be inflated by biparental inbreeding, are very low. Second, it shows that selfing was mostly absent not only in the last generation, as documented by the progeny-array estimates, but also in the few generations before, as attested by the population-level estimates which integrate selfing events over several generations (David *et al.*, 2007; Jarne and David, 2008; Wang *et al.*, 2012). Third, the sampled snails are most likely part of a single, coherent population, given that population-level selfing rate estimates do not suffer from a Wahlund effect. And fourth, our progeny-array estimates reflect "primary" selfing rates estimated in embryos, while the population-level estimates were computed using adult individuals that are, by definition, the surviving subset of all the snails originally present in the population. In principle, finding that "primary" and adult-level selfing rate

estimates are very similar thus indicates that in- or outbreeding depression is absent or weak (Ritland, 1990). However, as all four embryonic cohorts were devoid of selfed individuals, no inference can be made about the presence of in- or outbreeding from our study.

We conclude that RMES proves to be a valid alternative to the progeny-array approach for estimating selfing rates under a number of conditions, listed without a claim to completeness in Table 5 (see also Jarne and David, 2008; Wang *et al.*, 2012). From this list it is evident that using RMES as a fully equivalent method to progeny arrays puts relatively high requirements on the prior knowledge available about the population to be studied (conditions 1-5). Studies that unwittingly fail to meet one or several of these requirements run the risk of falsely inferring either selfing or its absence, depending on the assumptions that are not met. Progeny arrays should thus be used for estimating the selfing rate in populations that have not been studied before in sufficient detail, or if conditions 6 or 7 are not fulfilled. If all conditions are met, however, the savings in terms of time and money associated with using a population-level method are substantial, as for example in our study population-level estimates were computed using 5.7x fewer genotypes than progeny-array estimates (181 vs. 1034).

To the best of our knowledge, ours is only the third study in which selfing rates have been estimated simultaneously using the population-level methods implemented in RMES and using progeny arrays. In four populations of the terrestrial snail *Arianta arbustorum*, analyzed using four polymorphic microsatellite loci in 6-7 laboratory-bred families per population (mean 42 hatchlings per family), both methods yielded similar values, with progeny-array estimates exceeding RMES estimates only by 0.01, 0.03, 0.07 and 0.11, respectively (Kupfernagel *et al.*, 2010). Also in three populations of the coral *Favia fragum* both methods have been applied (Carlon and Lippé, 2011). Unfortunately, statistical power was very low in this dataset, caused by extremely low levels of heterozygosity within mothers (only 4/22 mothers heterozygous for ≥ 1 locus) despite an average of 13.7 polymorphic microsatellite loci per population. Undoubtedly, more studies are necessary until the biological and technical conditions will be identified under which RMES estimates can replace progeny-array estimates. Past studies on mating systems in hermaphroditic animals showed much variability among selfing rates (Jarne and Auld, 2006), and our empirical, field-based results support the previously voiced belief that unfortunately a significant part of this variation may stem from technical problems associated with the performance of the molecular markers that are available (Jarne and Auld, 2006; Jarne and David,

2008; Escobar *et al.*, 2011). Below we highlight this issue by commenting on previous studies evaluating the mating system of *Radix balthica*, some of which were done in our own research group.

Based on our study this natural snail population is fully outcrossing and does not show any variation in selfing rates, neither among families, nor during the reproductive lifespan within a single generation, nor across at least three consecutive generations. We accounted for a wide range of potential confounding effects, including biases due to null alleles, and ensured that the statistical power of our genetic markers was sufficient. We are thus confident in concluding that the mating system of *R. balthica* in the study population is, at least for the time being, stably outcrossing. This finding is largely in accordance with an early, F_{IS}-based analysis of four populations in Lake Geneva (Jarne and Delay, 1990).

The mating system of snail populations in Lake Zurich has been studied already before in our research group and the data have implied mixed mating (Wiehn *et al.*, 2002; Jokela *et al.*, 2006). In the year 2000, the same population was reported to be mixed-mating both based on progeny arrays and heterozygote deficiency in adults (Jokela *et al.*, 2006). Selfing had also been described to be widespread in four additional populations of *R. balthica* in Lake Zurich (Wiehn *et al.*, 2002). Clearly, these results are in stark contrast to our findings. Several

reasons for the discrepancy can be imagined. Differences to earlier, laboratory-based studies could have arisen where in the laboratory setting some isolated mothers ran out of allosperm. Alternatively, there could be true temporal or spatial differences in the mating system, for instance caused by bottlenecks and the ensuing scarcity of mating partners. At least for the study population this explanation appears unlikely, as population density has remained constant during the last two decades (J. Jokela, unpublished data). A long population history and ample time for accumulating mutations are also suggested by discontinuing allele size distributions at several microsatellite loci (e.g. loci Rb_1-3, Rb_8 and Rb_10; Table 4). Theoretically, discontinuing allele size distributions could also have arisen through hybridization of closely related *Radix* species, but the evident purity of the study population (see Supplementary Methods 1) speaks against this scenario. We thus consider it likely that two methodological issues led, together or separately, to an overestimation of selfing rates. The earlier studies, even if they used state-of-the-art methods at that time, relied on a small number of weakly polymorphic allozyme markers, resulting in insufficient statistical power for detecting outcrossed offspring (Bernatchez and Duchesne, 2000; Jarne and David, 2008). The low number of loci and low degree of polymorphism also led to difficulties in detecting null alleles, which might have led to heterozygote

547 deficiency (i.e. upwards biased F_{IS}-values) (David *et al.*, 2007; Jarne and
548 David, 2008; Wang *et al.*, 2012).

549 There is little evidence for selfing to be common in *R. balthica* also in two
550 more recent studies of molecularly identified, adult snails genotyped at eight
551 microsatellite loci and analyzed using RMES. Non-zero selfing rate estimates
552 were found in 16/25 European populations (Pfenninger *et al.*, 2011) and in 2/2
553 populations in western Switzerland (Haun *et al.*, 2012) that had sample sizes of
554 at least 20 genotyped individuals (mean $s(g_2)$ across all 27 populations: 0.16,
555 range: 0.00-0.53, mean sample size per population: 28.0). As no confidence
556 intervals are reported for these estimates, however, we do not know whether
557 some of them are significantly different from zero. The occurrence of mixed
558 mating in other populations of *R. balthica* thus cannot be confirmed until more
559 populations are analyzed using both reliable methods and adequate sample
560 sizes.

561 In conclusion, our study emphasizes the technical challenges in mating system
562 analysis. The positive note is that at present powerful methods are available for
563 both family- and population-level analyses that should allow for an accurate
564 estimation of mating systems if used appropriately. Both approaches may also
565 be applied fruitfully using next-generation sequencing data such as large
566 numbers of SNPs. Unmet conditions such as the presence of biparental

inbreeding when using indirect methods will cause the same difficulties independent of whether SNPs or microsatellites are used, especially when mixed maters are studied. However, the high resolution provided by millions of SNPs will undoubtedly facilitate progeny arrays. Unfortunately, our results also indicate the problems that may have gone undetected in earlier published assessments of selfing rates. Science is self-correcting, and therefore we hope that our study motivates re-analysis and re-assessment of selfing rates in cases where we need to know the mating system of our study species and populations.

Acknowledgments

We thank the editor and three anonymous reviewers for helpful comments on earlier versions of this manuscript, Kirstin Kopp for assistance with the establishment of a fast, cost-effective genotyping routine, and Patrice David for support when using RMES. DNA fragments were analyzed for length polymorphisms using a 3730xl DNA Analyzer (Applied Biosystems) situated at the Genetic Diversity Centre (GDC), ETH Zurich.

Conflict of Interest

The authors declare no conflict of interest.

Data Archiving

Sequence data have been submitted to GenBank: Accession No. KX832428-KX832513. Microsatellite genotype data have been submitted to Dryad: doi XXX.

Supplementary information is available at Heredity's website.

References

Ahlgren J, Yang X, Hansson LA, Bronmark C (2013). Camouflaged or tanned: plasticity in freshwater snail pigmentation. *Biol Lett* **9:** 20130464.

Allard RW (1975). The mating system and microevolution. *Genetics* **79:** 115-126.

Avise JC, Mank JE (2009). Evolutionary Perspectives on Hermaphroditism in Fishes. *Sex Dev* **3:** 152-163.

Baker HG (1955). Self-compability and establishment after "long-distance" dispersal. *Evolution* **9:** 347-349.

Belkhir K, Borsa P, Chikhi L, Raufaste N, Bonhomme F (1996-2004). *GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations*. Laboratoire Génome, Populations, Interactions, CNRS UMR 5171, Université de Montpellier II, Montpellier, France.

611

612 Bernatchez L, Duchesne P (2000). Individual-based genotype analysis in
613 studies of parentage and population assignment: How many loci, how many
614 alleles? *Can J Fish Aquat Sci* **57:** 1-12.

615

616 Brönmark C, Lakowitz T, Hollander J, Fenton B (2011). Predator-Induced
617 Morphological Plasticity Across Local Populations of a Freshwater Snail. *PLoS*
618 *One* **6:** e21773.

619

620 Carlon D, Lippé C (2011). Estimation of mating systems in Short and Tall
621 ecomorphs of the coral *Favia fragum*. *Mol Ecol* **20:** 812-828.

622

623 Charlesworth D, Charlesworth B (1987). Inbreeding depression and its
624 evolutionary consequences. *Annu Rev Ecol Syst* **18:** 237-268.

625

626 Cordellier M, Pfenninger M (2009). Inferring the past to predict the future:
627 climate modelling predictions and phylogeography for the freshwater gastropod
628 *Radix balthica* (Pulmonata, Basommatophora). *Mol Ecol* **18:** 534-544.

629

630 Coutellec-Vreto MA, Madec L, Guiller A (1997). Selfing and biparental
631 inbreeding: a mating system analysis in *Lymnaea peregra* (Gastropoda:
632 Lymnaeidae). *Heredity* **79:** 277-285.

633

634 Dabrowski MJ, Bornelöv S, Kruczyk M, Baltzer N, Komorowski J (2015).
635 'True' null allele detection in microsatellite loci: a comparison of methods,
636 assessment of difficulties and survey of possible improvements. *Mol Ecol*
637 *Resour* **15:** 477-488.

638

639 Dakin EE, Avise JC (2004). Microsatellite null alleles in parentage analysis.
640 *Heredity* **93:** 504-509.

641

642 David P, Pujol B, Viard F, Castella V, Goudet J (2007). Reliable selfing rate
643 estimates from imperfect population genetic data. *Mol Ecol* **16:** 2474-2487.

644

645 Escobar J, Auld J, Correa A, Alonso J, Bony Y, Coutellec M-A *et al.* (2011).
646 Patterns of mating-system evolution in hermaphroditic animals: Correlations
647 among selfing rate, inbreeding depression, and the timing of reproduction.
648 *Evolution* **65:** 1233-1253.

649

650 Fisher RA (1941). Average excess and average effect of a gene substitution.
651 *Ann Eugen* **11:** 53-63.

652

653 Haun T, Salinger M, Pachzelt A, Pfenninger M (2012). On the Processes
654 Shaping Small-Scale Population Structure in *Radix balthica* (Linnaeus 1758).
655 *Malacologia* **55:** 219-233.

656

657 Jarne P, Auld JR (2006). Animals mix it up too: The distribution of self-
658 fertilization among hermaphroditic animals. *Evolution* **60:** 1816-1824.

659

660 Jarne P, Charlesworth D (1993). The evolution of the selfing rate in
661 functionally hermaphrodite plants and animals. *Annu Rev Ecol Syst* **24:** 441-
662 466.

663

664 Jarne P, David P (2008). Quantifying inbreeding in natural populations of
665 hermaphroditic organisms. *Heredity* **100:** 431-439.

666

667 Jarne P, Delay B (1990). Population genetics of *Lymnaea peregra* (Müller)
668 (Gastropoda: Pulmonata) in Lake Geneva. *J Molluscan Stud* **56:** 317-322.

669

670 Jokela J, Wiehn J, Kopp K (2006). Among- and within-population variation in
671 outcrossing rate of a mixed-mating freshwater snail. *Heredity* **97:** 275-282.

672

673 Jones O, Wang J (2010). COLONY: a program for parentage and sibship
674 inference from multilocus genotype data. *Mol Ecol Resour* **10:** 551-555.

675

676 Kopp K, Wolff K, Jokela J (2012). Natural range expansion and human-assisted
677 introduction leave different genetic signatures in a hermaphroditic freshwater
678 snail. *Evol Ecol* **26:** 483-498.

679

680 Kupfernagel S, Rusterholz HP, Baur B (2010). Variation in multiple paternity
681 and sperm utilization patterns in natural populations of a simultaneous
682 hermaphrodite land snail. *Biol J Linn Soc* **99:** 350-361.

683

684 Lawton SP, Lim RM, Dukes JP, Kett SM, Cook RT, Walker AJ *et al.* (2015).
685 Unravelling the riddle of *Radix*: DNA barcoding for species identification of

686 freshwater snail intermediate hosts of zoonotic digeneans and estimating their
687 inter-population evolutionary relationships. *Infect Genet Evol* **35:** 63-74.

688

689 Nei M (1978). Estimation of average heterozygosity and genetic distance from
690 a small number of individuals. *Genetics* **89:** 583-590.

691

692 Pfenninger M, Cordellier M, Streit B (2006). Comparing the efficacy of
693 morphologic and DNA-based taxonomy in the freshwater gastropod genus
694 *Radix* (Basommatophora, Pulmonata). *BMC Evol Biol* **6:** 100.

695

696 Pfenninger M, Salinger M, Haun T, Feldmeyer B (2011). Factors and processes
697 shaping the population structure and distribution of genetic variation across the
698 species range of the freshwater snail *Radix balthica* (Pulmonata,
699 Basommatophora). *BMC Evol Biol* **11:** 135.

700

701 Pompanon F, Bonin A, Bellemain E, Taberlet P (2005). Genotyping errors:
702 causes, consequences and solutions. *Nat Rev Genet* **6:** 847-846.

703

Ritland K (1990). Inferences about inbreeding depression based on changes of the inbreeding coefficient. *Evolution* **44:** 1230-1241.

Ritland K (2002). Extensions of models for the estimation of mating systems using n independent loci. *Heredity* **88:** 221-228.

Ritland K, Jain S (1981). A model for the estimation of outcrossing rate and gene frequencies using n independent loci. *Heredity* **47:** 35-52.

Schniebs K, Gloer P, Vinarski MV, Hundsdoerfer AK (2011). Intraspecific morphological and genetic variability in *Radix balthica* (Linnaeus 1758) (Gastropoda: Basommatophora: Lymnaeidae) with morphological comparison to other European *Radix* species. *J Conchol* **40:** 657-678.

Waits L, Luikart G, Taberlet P (2001). Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. *Mol Ecol* **10:** 249-256.

722 Wang JL, El-Kassaby YA, Ritland K (2012). Estimating selfing rates from
723 reconstructed pedigrees using multilocus genotype data. *Mol Ecol* **21:** 100-116.

724

725 Weir BS, Cockerham CC (1984). Estimating F-statistics for the analysis of
726 population structure. *Evolution* **38:** 1358-1370.

727

728 Wiehn J, Kopp K, Rezzonico S, Karttunen S, Jokela J (2002). Family-level
729 covariation between parasite resistance and mating system in a hermaphroditic
730 freshwater snail. *Evolution* **56:** 1454-1461.

731

732 Wright S (1969). *Evolution and the Genetics of Populations: The Theory of*
733 *Gene Frequency v. 2*. University of Chicago Press: Chicago.

734

735 Zar JH (1996). Confidence Limits for Population Proportions. In: Fisher S and
736 Snavely SL (eds) *Biostatistical Analysis*, 3 edn. Prentice-Hall, Inc.: New Jersey,
737 p 525.

738

739

740

Tables

Table 1

Empirical studies that estimated both population-level and progeny-array-based selfing rates in the same population(s) of hermaphroditic animals. N_{pop}: number of populations for which both types of estimates were obtained; Marker type: microsatellites (M) or allozymes (A); N_{marker}: mean number of polymorphic markers per population; N_{adults}: mean number of adult individuals used for estimating population-level selfing rates; $s(F_{IS})$: mean selfing rate per population estimated based on the single-locus (s) or multilocus (m) inbreeding coefficient; $s(g_2)$: mean selfing rate per population estimated using two-locus heterozygosity disequilibrium values, calculated using the software RMES (David *et al.*, 2007); Progeny type: progenies collected in the field or produced in the laboratory either by field-collected mothers kept in isolation or by adults subjected to a mating trial experiment; Progeny age: developmental stage at which progenies were genotyped; N_{prog}: mean number of progenies used for estimating progeny-array-based selfing rates; $N_{off/prog}$: mean number of offspring genotyped per progeny; s(PA): mean selfing rate per population estimated based on progeny arrays, either calculated as the proportion of selfed offspring (prop. selfed) or from single-locus (s) or multilocus (m) outcrossing rates obtained from the software MLTR (Ritland, 2002). Full references can be found in the Supplementary Material.

Species	N_{pop}	Marker type	N_{marker}	N_{adults}	$s(F_{IS})$	$s(g_2)$	Progeny type	Progeny age	N_{prog}	$N_{off/prog}$	s(PA)	Reference
Bryozoa												
Cristatella mucedo	1	M	3	40	0.63 (s)	-	isolation	released larvae	16.0	1.6	0.36 (prop. selfed)	Freeland *et al.*, 2003
Cnidaria												
Seriatopora hystrix	1	A	8	50	0.55 (m)	-	isolation	released larvae	6.0	39.5	0.47 (m)	Sherman, 2008
	1	M	7	149	0.00 (unknown)	-	isolation	released larvae	13.0	38.1	0.00 (m)	Warner *et al.*, 2016
Echinodermata												
Amphipholis squamata	2	M	1.7	54	1.0 (m & s)	-	field & isolation	newborns	25.3	3.4	1.0 (prop. selfed)	Boissin *et al.*, 2008
Mollusca												
Arianta arbustorum	4	M	4	6.5	0.04 (s)	0.08	isolation	hatchlings	6.5	42.2	0.13 (m)	Kupfernagel *et al.*, 2010
Bulinus truncatus	5	M	4	35	0.93 (m)	-	isolation	adults	11.4	7.9	0.85 (m)	Viard *et al.*, 1997
Biomphalaria pfeifferi	6	M	6	17.5	0.91 (m)	-	isolation	3 days after hatching	8.5	4.2	0.78 (m)	Charbonnel *et al.*, 2005
Lymnaea ovata (aka *Radix balthica*)	4	A	4-8	30	0.60 (m)	-	field & isolation	subadults & adults	9.0	16.0	0.64 (m)	Wiehn *et al.*, 2002
	2	A	3.5	63	0.29 (m)	-	isolation	hatchlings	26.5	15.0	0.75 (m)	Jokela *et al.*, 2006
Lymnaea truncatula	6	M	6.7	44.9	0.85 (m)	-	isolation	hatchlings	5.0	7.9	0.87 (m)	Trouvé *et al.*, 2003

(aka *Galba truncatula*)	2	M	2	30.5	0.88 (m & s)	-	mating trial	hatchlings	21.5	8.7	0.69 (m & s)	Meunier *et al.*, 2004
Physa acuta	1	A	3	30	0.06 (m)	-	isolation	3 weeks after hatching	30.0	5.6	0.08 (m)	Jarne *et al.*, 2000
	4	M	4.3	17.3	0.16 (m)	-	unknown	unknown	10.0	6.7	0.11 (m)	Bousset *et al.*, 2004
	11	M	6.5	16.1	0.11 (m)	-	isolation	7 days after hatching	9.6	6.7	0.06 (m)	Henry *et al.*, 2005

Table 2

Polymorphism data and population-level inbreeding coefficients of adult snails of the species *Radix balthica* collected on six days from a single natural population. Values are shown for analyses either excluding or including locus Rb_3, which showed a high frequency of null alleles. N: number of adult snails genotyped; N_P: number of polymorphic microsatellite loci; N_A: mean number of alleles per locus; H_O: mean observed heterozygosity; H_E: mean expected heterozygosity calculated without bias (Nei, 1978); F_{IS}: multilocus inbreeding coefficient according to Weir & Cockerham (1984) with a 95% confidence interval based on 10'000 bootstrap iterations. Numbers in bold are significantly different from zero.

		Without locus Rb_3						With locus Rb_3					
Sampling day	N	N_P	N_A	H_O	H_E	F_{IS}	CI95	N_P	N_A	H_O	H_E	F_{IS}	CI95
24.04.2013	85	9	11.4	0.662	0.707	**0.064**	[0.017, 0.099]	10	12.0	0.637	0.729	**0.127**	[0.080, 0.160]
06.03.2014	49	9	10.9	0.636	0.714	**0.109**	[0.046, 0.150]	10	11.3	0.611	0.735	**0.170**	[0.108, 0.210]
22.03.2014	58	9	9.7	0.630	0.677	**0.070**	[0.009, 0.115]	10	10.1	0.609	0.700	**0.130**	[0.071, 0.173]
11.04.2014	51	9	11.2	0.651	0.702	**0.074**	[0.010, 0.118]	10	11.9	0.633	0.724	**0.128**	[0.065, 0.171]
23.05.2014	23	9	8.2	0.673	0.720	0.067	[-0.060, 0.149]	10	8.7	0.653	0.740	**0.121**	[0.008, 0.186]
13.04.2015	50	9	10.9	0.693	0.720	0.039	[-0.015, 0.070]	10	11.1	0.652	0.740	**0.120**	[0.066, 0.153]

Table 3

Number and probability of being selfed among successfully genotyped embryos from field-collected egg clutches. Results are shown for analyses either excluding or including locus Rb_3, which showed a high frequency of null alleles. Individual probabilities of being selfed were estimated using COLONY (Jones and Wang, 2010). $N_{clutches}$: number of egg clutches from which embryos were genotyped; $N_{embryos}$: number of successfully genotyped embryos; Embryo ID: identity of embryos with probabilities of being selfed ≥0.01, written as "embryo number_clutch number"; P(selfed): individual probability of being selfed. Of embryos with P(selfed) ≥0.01, only one was undeveloped (13_c19 on sampling day 06.03.2014). This was also the only embryo with P(selfed) ≥0.01 in analyses both excluding and including locus Rb_3.

			Without locus Rb_3		With locus Rb_3	
Sampling day	$N_{clutches}$	$N_{embryos}$	Embryo ID	P(selfed)	Embryo ID	P(selfed)
06.03.2014	15	223	13_c19 all others	0.263 ≤0.007	13_c19 01_c09 07_c03 all others	0.340 0.179 0.028 ≤0.003
22.03.2014	15	219	all embryos	≤0.004	all embryos	≤0.003
11.04.2014	15	361	all embryos	≤0.004	43_c04 09_c32 20_c04 55_c04 20_c30 13_c04 all others	0.043 0.038 0.016 0.015 0.012 0.011 ≤0.007
23.05.2014	15	231	08_c25 08_c15 all others	0.388 0.062 0.000	04_c41 all others	0.439 ≤0.003

Table 4

Characterization of ten microsatellite loci for *R. balthica* based on adult snails and embryos from a single natural population. Shown is the locus name, repetitive sequence, accession number in Genbank, primer sequence, range of product sizes, number of observed alleles N_A (range within snails from a single sampling day, plus total number in brackets), and the range of expected (H_E) and observed (H_O) heterozygosity values. Ranges of N_A, H_E and H_O are provided for adults (A) and embryos (E) separately. Only a single embryo was taken per clutch to forestall any bias caused by sibship, resulting in a total of 15 embryos per sampling day. To ensure comparability, groups of adults were restricted to a random sample of 15 snails per sampling day, too. The last column provides the rates of inferred genotyping errors caused by null alleles (top) and other kinds of errors (bottom). These rates were used in parentage analyses, and were computed based on a larger sample of snails, including but not restricted to the individuals genotyped for this study (2044 genotypes in total, see Methods for details).

Locus	Repeat motif	Accession no.	Primer sequence (5'-3')	Size (bp)	N_A	H_E	H_O	Inferred genotyping error rates
Rb_1	$(TG)_{12}$	KX830983	F: AACCTTGGGATGGAAGTCGG R: CCAAACTACCGAACAGAGCG	227-309	A: 6-9, E: 5-9 (25)	A: 0.584-0.798 E: 0.559-0.662	A: 0.533-0.800 E: 0.333-0.667	0.0533 (null alleles) 0.0083 (other errors)
Rb_2	$(ATAG)_8$	KX830984	F: ACGTCATGCTCGTTTGGATG R: ACTTCCCCAACTGATCTCGG	155-253	A: 3-4, E: 3-4 (7)	A: 0.522-0.653 E: 0.191-0.678	A: 0.333-0.667 E: 0.200-0.733	0.0602 0.0054
Rb_3	$(AGAT)_{20}$	KX830985	F: AAGGAAGACAGACAGACGGG R: TGATACGATCTGTGCCAATCAG	121-229	A: 9-11, E: 9-13 (22)	A: 0.878-0.915 E: 0.892-0.926	A: 0.267-0.733 E: 0.200-0.600	0.0822 0.0470
Rb_4	$(ACAT)_8$	KX830986	F: ACGTCATATCCGTTACATCCAC R: GCGGTCAGTCTTTGATCTCC	159-196	A: 4-7, E: 4-5 (9)	A: 0.538-0.694 E: 0.435-0.639	A: 0.467-0.733 E: 0.200-0.800	0.0401 0.0029
Rb_5	$(AACA)_8$	KX830987	F: ACATCCTGATCCAGAAGTAACATAAAG R: TCACATTTCGAGAACGAGGC	122-166	A: 6-8, E:5-7 (10)	A: 0.789-0.832 E: 0.770-0.828	A: 0.733-0.867 E: 0.600-0.933	0.0303 0.0225
Rb_6	$(ATGT)_{13}$	KX83098	F: AATGCGTAAGGCGAGGGTAG	228-313	A: 4-11, E: 5-9	A: 0.706-0.862	A: 0.400-0.733	0.0802

		8	R: TTCACATGATGACAGACATTCC		(16)	E: 0.685-0.848	E: 0.333-0.600	0.0068
Rb_7	$(AGAT)_{22}$	KX83098 9	F: ACGGGGGTAATTCGTCATGC R: TCCATCCCCACACATACACG	203-279	A: 12-13, E: 11-14 (19)	A: 0.906-0.936 E: 0.832-0.933	A: 0.800-1.000 E: 0.800-1.000	0.0372 0.0117
Rb_8	$(TCTA)_{17}$	KX83099 0	F: CTCTGTGTGCCTGCCTGC R: ACTACCAAACTTACACATGCAC	117-293	A: 8-12, E: 8-12 (29)	A: 0.841-0.894 E: 0.860-0.922	A: 0.667-0.933 E: 0.800-0.933	0.0284 0.0391
Rb_9	$(TCTA)_{14}$	KX83099 1	F: TCTCTTTCTCTATCTTTCCCACCC R: AGCCAATGAAGAGAGGTATGG	176-274	A: 8-13, E: 10-11 (23)	A: 0.777-0.908 E: 0.830-0.867	A: 0.733-0.933 E: 0.733-0.933	0.0572 0.0186
Rb_10	$(CTA)_{7}$	KX83099 2	F: CCTACCACGTCCACCACTAC R: AGAGAAAAGAACAGCAAAAGAACC	129-201	A: 3-7, E: 1-6 (14)	A: 0.191-0.552 E: 0.000-0.414	A: 0.133-0.533 E: 0.000-0.400	0.0455 0.0039

785

Table 5

Conditions for using the software RMES (David *et al.*, 2007) as a fully valid alternative to progeny arrays when estimating selfing rates. This list is not intended to be exhaustive (see also Jarne and David, 2008; Wang *et al.*, 2012). For further information, see Discussion.

#	Condition
1	Biparental inbreeding absent
2	Selfing rates stable over time
3	Population not subdivided
4	Allele and genotype frequencies at equilibrium
5	One or several of the following: - In- or outbreeding depression absent - In- or outbreeding depression present, but strength known from earlier studies (allowing adult-based estimates to be corrected) - In- or outbreeding depression irrelevant because of absence of selfing already in embryos - In- or outbreeding depression irrelevant because selfing shall be quantified explicitly at later stages of life
6	Selfing to be detected across several preceding generations
7	Variation in selfing rates between individuals not of interest

Titles and legends to figures

Figure 1

Selfing rates in a natural population of the freshwater snail *R. balthica*, estimated using one direct and three indirect methods and separately assessed on six sampling days spanning three years. Error bars show 95% confidence intervals. Selfing rates were computed using nine highly polymorphic microsatellite loci that are largely free from null alleles (a), and including an additional locus with a high frequency of null alleles (b). Selfing rates were obtained directly from field-collected egg clutches (progeny-array method yielding "primary" selfing rates; 15 egg clutches with a total of 259 ± 34 (mean ± SE) embryos for each of four sampling days) and indirectly from field-collected adult snails (population-level methods; 52.7 ± 8.1 adults for each of six sampling days). Population-level selfing rates were estimated in three ways: from the multilocus inbreeding coefficient F_{IS} (Weir and Cockerham, 1984) using the relationship $s=2F_{IS}/(1+F_{IS})$ (Wright, 1969), from two-locus heterozygosity disequilibrium values (g_2) using RMES (David *et al.*, 2007), and by maximizing the log-likelihood of the multilocus heterozygosity structure of the sample (ML), also using RMES.

Figures

Figure 1