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1 Grandparent inference from genetic data: The potential for parentage-based tagging programs to
2 identify offspring of hatchery strays

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14 Running head: Grandparent-grandchild trio inference

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16 Abstract

17 Fisheries managers routinely use hatcheries to increase angling opportunity. Many
18 hatcheries operate as segregated programs where hatchery-origin fish are not intended to spawn
19 with natural-origin conspecifics in order to prevent potential negative effects on the natural-
20 origin population. Currently available techniques to monitor the frequency with which hatchery-
21 origin strays successfully spawn in the wild rely on either genetic differentiation between the
22 hatchery- and natural-origin fish or extensive sampling of fish on the spawning grounds. We
23 present a method to infer grandparent-grandchild trios using only genotypes from two putative
24 grandparents and one putative grandchild. We developed estimators of false positive and false
25 negative error rates and showed that genetic panels containing 500 - 700 single nucleotide
26 polymorphisms or 200 - 300 microhaplotypes are expected to allow application of this technique
27 for monitoring segregated hatchery programs. We discuss the ease with which this technique can
28 be implemented by pre-existing parentage-based tagging programs and provide an R package
29 that applies the method.

30

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31 Introduction

32 Fisheries managers have long used hatcheries to increase angling opportunity and to
33 compensate for anthropogenic impacts that have decreased population sizes (Waples et al. 2007).
34 In some situations, it has been observed that hatchery-origin fish have lower fitness in the wild
35 relative to natural-origin conspecifics, potentially due to selection in the hatchery environment or
36 the use of hatchery strains that are not locally adapted (Ford 2002; Miller et al. 2004; Araki et al.
37 2007a, 2007b, 2008; Christie et al. 2014). In order to prevent negative effects of hatchery- and
38 natural-origin fish interbreeding, it has been recommended that hatcheries operate as either
39 integrated or segregated programs (Hatchery Scientific Review Group 2009). Integrated
40 programs aim to balance the proportion of natural-origin fish in the hatchery broodstock and the
41 proportion of hatchery-origin fish spawning naturally to minimize the effect of domestication
42 (Goodman 2005). Segregated programs intend to minimize gene flow between hatchery- and
43 natural-origin populations (Mobrand et al. 2005; Hatchery Scientific Review Group 2009). These
44 strategies, developed in the context of Pacific salmon hatcheries, represent alternative
45 approaches to both protect natural-origin populations and provide harvest opportunities for
46 anglers.

47 In order to evaluate the efficacy of segregated hatchery programs, a method of
48 monitoring gene flow from hatchery-origin fish to nearby natural-origin populations is needed.
49 This has been previously estimated in several ways. Some studies estimate the proportion of fish
50 on the spawning grounds that are of hatchery origin (pHOS) through observation of marks and/or
51 tags present on hatchery fish (Dauer et al. 2009; Tattam and Ruzycki 2020). This metric is
52 important as it demonstrates the potential for competition between hatchery- and natural-origin
53 fish on the spawning grounds and suggests that hatchery introgression may be occurring.

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54 Alternatively, if the hatchery- and natural-origin populations show sufficient genetic
55 differentiation, migration rates can be estimated from temporal samples (van Doornik et al.
56 2013), or genetic structure of hatchery- and natural-origin populations can be evaluated to
57 determine whether the pattern indicates hatchery introgression (Matala et al. 2012; Ozerov et al.
58 2016; Lehnert et al. 2020). All of these approaches have drawbacks. Observing the proportion of
59 hatchery-origin fish on the spawning grounds, while suggestive, does not directly assess gene
60 flow as the reproductive success of hatchery-origin fish is unknown. Methods utilizing genetic
61 differentiation are not applicable to cases without sufficient differentiation between the hatchery-
62 and natural-origin populations, which is common where hatchery stocks were derived from
63 nearby natural-origin populations.

64 An alternative technique uses genetic samples to infer relationships between hatchery
65 broodstock and individuals sampled in the wild. Hatchery broodstock can be genetically sampled
66 at the time of spawning, and their genotypes later used to infer whether a given fish is a
67 descendent of hatchery broodstock. Parentage-based tagging (PBT) uses this approach to identify
68 offspring of the hatchery broodstock for monitoring and management of hatchery stocks
69 (Anderson and Garza 2005, 2006). Parentage-based tagging has been implemented and validated
70 on large and small scales for a variety of species (DeHaan et al. 2008; Denson et al. 2012; Evans
71 et al. 2018; Bingham et al. 2018; Campbell et al. 2019; Vandeputte et al. 2021), most notably
72 Pacific salmonids (Steele et al. 2013, 2019; Beacham et al. 2019).

73 With appropriate methods for statistical inference, the general approach of PBT can be
74 extended to identify grandchildren of hatchery broodstock. Genetic samples can be taken from
75 hatchery broodstock, and samples from natural-origin fish can later be assessed to determine if
76 they are grandchildren of those broodstock (and therefore had an unsampled, hatchery-origin

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77 parent). The relationship being inferred is a grandparent-grandchild trio consisting of one
78 grandchild and two grandparents on the same side (i.e., either both maternal or both paternal
79 grandparents). The other two grandparents and the parents are unsampled and therefore have
80 unknown genotypes (Figure 1). While comprehensive sampling in the hatchery is
81 straightforward, similar sampling of adults spawning naturally is often logistically prohibitive.
82 The ability to infer recent hatchery ancestry without sampling naturally spawning parents would
83 overcome this issue.

84 Methods have previously been developed for inferring grandparent-grandchild
85 relationships, but these methods are not optimal for inferring grandparent-grandchild trios.
86 Letcher and King (2001) describe a method for inferring the relationship between all four
87 grandparents and a grandchild. To identify offspring of hatchery strays, this method requires
88 sampling natural-origin grandparents as well as hatchery broodstock which is not feasible in
89 many situations. Christie et al. (2011) describe a method to infer grandparent-grandchild trios by
90 identifying trios with no observed Mendelian incompatibilities (or less than a specified number).
91 While this method has been successfully applied to situations where one parent is known
92 (Christie et al. 2011; Sard et al. 2016), the lower power of exclusionary methods compared to
93 likelihood-based methods (Anderson and Garza 2006) make exclusionary methods less feasible
94 when no parents are sampled.

95 We here report a likelihood-based method to identify grandparent-grandchild trios using
96 genotypes. Additionally, we develop techniques to estimate assignment error rates for this
97 method and provide an R package implementing the described techniques
98 (https://github.com/delomast/gRandma).
99

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100 Methods

101 *Inferring grandparent-grandchild relationships*

102 Relationships have previously been inferred from genetic data using likelihood ratios to
103 compare the putative relationship (e.g. parent-offspring) to an alternative relationship (typically
104 that the individuals are unrelated) (Marshall et al. 1998; Anderson and Garza 2006; Kalinowski
105 et al. 2007; Anderson 2012). Methods for calculating likelihoods of relationships (simple
106 pedigrees) have previously been detailed (Thompson 1976, 2000; SanCristobal and Chevalet
107 1997; Anderson and Garza 2006), and we extended this approach specifically to grandparent-
108 grandchild trios. Given allele frequencies for a locus under Hardy-Weinberg equilibrium (HWE),
109 the likelihood of the three individuals being unrelated was calculated as the product of the
110 probability of sampling each genotype from the population. The likelihood of the individuals
111 being a grandparent-grandchild trio was calculated by utilizing allele frequencies and the laws of
112 Mendelian inheritance. Genotyping error was accounted for by marginalizing the true genotypes
113 utilizing estimates of genotyping error (Anderson and Garza 2006). This allows genotyping error
114 to be flexibly modeled in any way that yields, for a given true genotype, the probability of
115 observing each genotype. With the R package we provide, users can specify these probabilities or
116 utilize a default error model. The default error model, used in the analyses described in this
117 study, is described in supplementary file 1. Loci were considered to be independent, as in
118 previous methods of relationship inference and pedigree reconstruction (Marshall et al. 1998;
119 Riester et al. 2009; Jones and Wang 2010; Anderson 2012; Huisman 2017).

120 Calculating likelihoods for large numbers of possible trios can be computationally
121 intensive. We therefore implemented a preliminary screen based on the observed number of
122 Mendelian incompatibilities (MI) in a grandparent-grandchild trio. Trios with more than m MIs

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123 were excluded from consideration. The value of m was chosen so that the probability of
124 excluding a true grandparent-grandchild trio was less than 0.0001 for a trio with no missing
125 genotypes. With a constant value of m, the probability of rejecting a trio with one or more
126 missing genotypes is smaller, as a locus with a missing genotype cannot be considered a
127 Mendelian incompatibility. The probability of rejecting a true trio given a value of m was
128 calculated by representing the number of MIs as a Markov Chain, following the method detailed
129 by Anderson (2012) but extended to the relationship of grandparent-grandchild trio.
130 Assigning grandparent-grandchild relationships can be treated as a hypothesis test by
131 comparing the calculated log-likelihood ratio (LLR) to a critical value, c (SanCristobal and
132 Chevalet 1997; Anderson and Garza 2006). If the LLR was greater than or equal to c, the trio
133 was considered related; otherwise, the trio was considered unrelated. The value of c can be
134 chosen to achieve a desired balance of false negative and false positive error rates.
135
136 *False positive error rates*
137 Per-comparison false positive error rates (probability of assigning a relationship to a trio
138 that is not a grandparent-grandchild trio) are dependent upon the true relationship for a trio. We
139 have implemented methods to assess false positive error rates for unrelated trios (typically the
140 most important) as well as 13 other types of relationships. In these relationships, the two putative
141 grandparents were considered unrelated to each other, but the putative grandparents had different
142 relationships to the putative grandchild. The relationships considered represented combinations
143 of true grandparents, individuals unrelated to the putative grandchild, great-aunts, half-great-
144 aunts, and first cousins of the putative grandchild's true grandparent.
145 Estimating the per-comparison false positive error rate for a given value of c has been

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146 demonstrated for parent-offspring and sibling relationships using importance sampling
147 (Anderson and Garza 2006; Baetscher et al. 2018), a Monte Carlo variance reduction technique.
148 Variance reduction is needed as a naïve Monte Carlo approach would be inefficient at estimating
149 very small false positive rates (Anderson and Garza 2006). Small error rates can be meaningful
150 as the experiment-wide false positive error rate is estimated by the product of the per-comparison
151 false positive error rate and the number of trios (with the corresponding true relationship)
152 considered. We extended this approach to the current application of assessing grandparent-
153 grandchild trios. We also implemented an alternative method utilizing stratified sampling,
154 another Monte Carlo variance reduction technique, because specific implementations of
155 importance sampling can produce unreliable results (Owen 2013).
156
157 *Importance sampling*
158 A standard Monte Carlo estimator of false positive rates would be to simulate genotypes
159 for trios of a given relationship (such as unrelated) and record how many of them fit the criteria
160 to be considered related. Importance sampling can be thought of as focusing the simulation on
161 producing mostly genotypes that do assign and then correcting for this modification. To
162 implement importance sampling, genotypes were simulated from the distribution of genotypes in
163 true grandparent-grandchild trios. Missing genotypes were accounted for utilizing the forward-
164 backward algorithm described below with the state of the Markov chain representing the number
165 of missing genotypes in one individual. If the simulated trio had fewer than m MIs and the
166 calculated LLR was greater than or equal to c, the observation was recorded as a false positive
167 with the appropriate importance sampling weight (Owen 2013).
168

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169 *Stratified sampling*

170 Similar to importance sampling, the goal of stratified sampling was to focus simulation
171 effort on categories (strata) that produce false positives. We stratified the distribution of trio
172 genotypes by the number of observed MIs. This was a natural choice because the algorithm we
173 use explicitly filters possibilities based on the number of observed MIs. Therefore, we can
174 eliminate simulating genotypes for most strata. Sampling effort can then be focused on trios with
175 m or fewer MIs. This method requires calculating the probability that a trio of given relationship
176 has a given number of observed MIs and the ability to simulate genotypes for a trio given a
177 relationship and number of MIs. Genotypes for trios were simulated in each stratum and the false
178 positive rates were recorded. Utilizing the probabilities that a trio has each number of MIs (i.e.,
179 the size of the strata), the overall false positive rate was then calculated.

180 To calculate the probability a trio has a given number of observed MIs, we represented
181 the observation of MIs and missing genotypes as a Markov chain and utilized the forward step of
182 the forward-backward algorithm. We extended the approach described by Anderson (2012) to
183 account for the common practice of only analyzing samples given a maximum number of
184 missing genotypes and to fit the target relationships. The maximum number of missing
185 genotypes allowed in the current analyses was 10% of loci. Let s_i be the state, describing the
186 number of observed MIs and missing genotypes, after observing locus i. Prior to observing any
187 loci, $s_0 = (0,0,0,0)$, representing the number of observed incompatibilities and number of
188 missing genotypes for the three individuals. Let a_i be a vector indicating whether an
189 incompatibility or any missing genotypes are observed at locus i, in the same order as s_i.
190 Assuming HWE, known allele frequencies, known probability of a genotype being missing, and
191 observation of missing genotypes is independent across loci and individuals, the probabilities of

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192 each possibility for a_i can be calculated according to standard probability arguments for a given
193 true relationship. The probability of being in state x after a given locus can then be calculated as

$$\mathrm{P}(\mathrm{s}_{\mathrm{i}+1} = x) = \mathrm{P}(\mathrm{s}_{\mathrm{i}}) \sum_{\mathrm{a}} \mathrm{P}(\mathrm{a}_{\mathrm{i}+1}) \mathrm{I}\{\mathrm{s}_{\mathrm{i}} + \mathrm{a}_{\mathrm{i}+1} = x\}. \quad (1)$$

194 This can be evaluated recursively to obtain the probabilities of each final state. The probability a
195 trio has a given number of observed MIs can then be calculated given a maximum number of
196 missing genotypes. However, memory constraints can make saving all the probabilities at each
197 step impractical even with moderate numbers of loci. The probability of observing an individual
198 with more than the allowed number of missing genotypes can be obtained through the same
199 algorithm, but with s and a now only representing missing genotypes in one individual. Because
200 we assumed that missing genotypes are independent between individuals, the probability of all
201 three samples having a valid number of missing genotypes is then straightforward to calculate
202 and only the probabilities of states with m or fewer MIs and with all three individuals having an
203 allowable number of missing genotypes need to be saved.

204 To utilize stratified sampling, genotypes need to be simulated for trios with a specified
205 number of MIs. The backwards step of the forward-backward algorithm fills this need. Given L
206 loci, a value of s_L is chosen given the number of MIs by sampling a categorical distribution with
207 probabilities proportional to the probability of each s_L that has the specified number of MIs (and
208 allowable number of missing genotypes). Next, for each locus and iterating backwards, a value
209 for a_i is chosen by sampling a categorical distribution with

$$P(a_i | s_{i+1}) \propto P(a_i) P(s_i = s_{i+1} - a_i). \quad (2)$$

210 Once a_i is chosen, genotypes are sampled using the genotype frequencies for the true
211 relationship calculated from the allele frequencies, HWE, laws of Mendelian inheritance, and
212 genotyping error rates. If all three genotypes are observed (i.e., no missing genotypes in the

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213 chosen a_i), then the genotypes are either sampled conditional upon an MI being present or not.

214

215 *False negative error rate*

216 To estimate the per-comparison false negative error rate (probability of failing to assign a

217 relationship to a true grandparent-grandchild trio) for a given value of *c*, Monte Carlo methods

218 have been previously used for other relationships (Anderson and Garza 2006; Baetscher et al.

219 2018) and we adopted this strategy here. Genotypes of grandparent-grandchild trios are

220 simulated and loci with missing genotypes for each individual are chosen by representing the

221 observation of missing genotypes as a Markov chain and utilizing the forward-backward

222 algorithm, as described above with the state representing the number of missing genotypes in one

223 individual. Log-likelihood ratios were calculated for the simulated genotypes, and the proportion

224 of trios with the number of MIs greater than *m* or LLR less than *c* was the estimate of the false

225 negative rate.

226

227 *Panel size simulations and error rate estimator evaluation*

228 A key question for designing experiments implementing this method is, how many loci

229 need to be genotyped to obtain reliable assignments? To help answer this question, we estimated

230 error rates for panels of different size. We simulated panels containing 100, 300, 500, 700, and

231 900 biallelic single nucleotide polymorphisms (SNPs) and panels containing 100, 200, 300, and

232 400 triallelic microhaplotypes. The SNPs and microhaplotypes were simulated with expected

233 heterozygosity of 0.22 (allele frequencies of 0.125 and 0.872) and 0.42 (allele frequencies of

234 0.13, 0.13, and 0.74), respectively. This choice reflects the mean expected heterozygosities for

235 SNPs and microhaplotypes in a comparison of both marker types for relationship inference in

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236 rockfish (Baetscher et al. 2018). The probability of a missing genotype at a locus was set at 3%
237 in these analyses. It is common practice to remove any samples with more than a threshold
238 number of missing genotypes, and so we restricted all simulated genotypes to have 90% or more
239 genotypes present. False negative and false positive, given a true relationship of unrelated, error
240 rates were estimated for a range of c values and the relationship between error rates was
241 compared between panels. Integer values of c were chosen starting at 0 and increasing until the
242 estimated false negative rate was above 0.05. Estimates of false negative rates and estimates
243 from the importance sampling method were derived from 10,000 and 1,000,000 Monte Carlo
244 iterations, respectively. Estimates from the stratified sampling routine were derived from
245 1,000,000 iterations for each stratum (number of observed Mendelian incompatibilities) less than
246 or equal to m.

247 To compare performance of the two methods for estimating false positive error rates, the
248 estimated error rates for unrelated trios were compared between the methods with all four
249 simulated microhaplotype panels. Estimates for other true relationships were compared using the
250 300 locus microhaplotype panel. Finally, to examine the importance of modelling the presence of
251 missing genotypes, we compared importance sampling estimates using the 300 locus
252 microhaplotype panel with the probability of a genotype being missing equal to 3% and 0%.

253 The scripts used to perform these simulations and their outputs are available at
254 https://github.com/delomast/gpError2021.

255

256 *Example analyses*

257 To fully evaluate false positive per-comparison error rates, one needs to have a general
258 idea of the size of the analysis (number of comparisons) being attempted. We consider two

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259 examples modeled around steelhead *Oncorhynchus mykiss* hatchery programs in the Snake River
260 basin using data collected during 2018. The Upper Salmon B-run (USB) represents a smaller
261 hatchery program and spawned 66 steelhead in 2018. The Dworshak National Fish Hatchery
262 (DNFH) represents a larger hatchery program and spawned 1,778 steelhead in 2018. Both of
263 these hatchery programs take genetic samples from all broodstock and record the day of
264 spawning, phenotypic sex, and crosses made. Data collected at the hatchery (or a genetic sex
265 marker) can be used to constrain the number of possible pairs of grandparents considered in an
266 analysis. For each hatchery program, we consider the effect on the desired per-comparison false
267 positive rate of using no data, phenotypic sex, spawn day, phenotypic sex and spawn day, or
268 cross records.

269 In the example analysis, we assume that natural-origin juveniles are sampled and that
270 exact age is unknown but is constrained to one, two, or three years old. The effect of this
271 assumption is that three potential years of parents must be considered for each juvenile.
272 Hatchery-origin steelhead in the Snake River basin return almost exclusively as three and four
273 year old fish (Warren et al. 2017), so this translates to four years of potential grandparents that
274 must be considered.

275 The total number of comparisons in these analyses is the product of the number of
276 possible pairs of grandparents per year, the number of years of potential grandparents being
277 considered (four), and the number of potential grandchildren evaluated (assumed here to be 200).
278 We then calculate the desired per-comparison false positive (true relationship of unrelated) error
279 rate to achieve an expected number of false positive assignments of 0.1 (0.1 / number of
280 comparisons) assuming all trios are unrelated. This ignores false positives arising from trios of
281 other relationships. In some situations, error rates for alternative relationships are important to

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282 consider, but in analyses of segregated hatchery programs (where no breeding of hatchery-origin
283 and natural-origin fish is desired), false positives arising from trios of other relationships are not
284 necessarily harmful as the purpose is to identify fish with recent hatchery-origin ancestry.
285 Additionally, the vast majority of trios considered are likely unrelated, and so the false-positive
286 rate for unrelated trios will often be the most impactful.
287
288 Results
289 Estimated error rates declined with increasing panel size, and the microhaplotype panels
290 showed lower error rates than SNP panels of similar size (Figure 2). For example, false positive
291 error rates (true relationship of unrelated) below 10^{-10} were achieved at false negative error rates
292 below 0.05 with SNP panels containing 700 or more loci and microhaplotype panels containing
293 300 or more loci. False positive rates for related trios decreased with decreasing relatedness
294 between individuals in the trio (Supplemental Figure 1).
295 False positive rates estimated by importance sampling and stratified sampling were
296 practically identical when estimates were above approximately 10^{-8} (Figure 3). As the false
297 positive rate decreased below this, the importance sampling method estimated false positive rates
298 higher than the stratified sampling method. In these cases, the stratified sampling method
299 estimated false positive rates of 0 within one or more strata (i.e., no false positives were sampled
300 out of the 1,000,000 iterations). Similar results were obtained for false positive rates estimated
301 for trios with relationships other than unrelated (Figure 4) except for two trio types (true
302 grandparent and unrelated; true grandparent and cousin of grandparent) that had noticeably
303 different estimates between the two methods and did not have a low false positive rate.
304 Incorporating a 3% missing genotype rate in the error rate estimation had a moderate

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305 effect on the results (Figure 5). For example, at a false negative rate of 0.05, the false positive
306 (true relationship of unrelated) error rates were approximately (derived from linear interpolation
307 with neighboring points) $1.3 \cdot 10^{-11}$ and $2.1 \cdot 10^{-12}$ when missing genotypes had rates of 3% and
308 0%, respectively.

309 The example hatchery programs considered show that analysis of smaller programs (e.g.,
310 USB) require per-comparison false positive rates on the order of 10^{-6} - 10^{-8} depending on what
311 data, if any, is available to reduce the number of comparisons (Table 1). Larger programs, similar
312 to DNFH, require rates on the order of 10^{-7} - 10^{-10} if the number of comparisons can be reduced
313 by one of the data sources considered. Error rates estimated for the simulated panels (Figure 2)
314 suggest that these rates can be achieved with panels containing 500 - 700 SNPs or 200 - 300
315 microhaplotypes. If no data is available to reduce the number of pairs of grandparents that must
316 be considered, then hatchery programs similar in size to DNFH (1,700 fish spawned / year) will
317 require error rates on the order of 10^{-11}, which is achievable with panels containing 700 - 900
318 SNPs or 300 - 400 microhaplotypes.

319

320 Discussion

321 The methods developed here facilitate direct monitoring of introgression between
322 hatchery- and natural-origin populations without relying on genetic differentiation. This fills an
323 unmet need for monitoring the segregated hatchery programs upon which many fisheries rely.
324 The method identifies fish with an unsampled, hatchery-origin parent using genetic samples from
325 the hatchery broodstock and estimates error rates given allele frequencies for a population.

326 Another benefit of this method is the ease with which it can be implemented through
327 existing PBT programs because the hatchery and laboratory processes required to sample and

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328 genotype broodstock are already in place. Additional requirements for grandparent inference
329 would be the development of a genetic panel with sufficient power (given the size of the relevant
330 hatcheries) and collection of samples from natural-origin fish that are potential grandchildren of
331 the hatchery broodstock. In the past decade, there have been multiple, large-scale demonstrations
332 of the efficacy of PBT for monitoring fisheries (Steele et al. 2013, 2019; Beacham et al. 2019).
333 Numerous PBT programs have been recently reported (Evans et al. 2018; Bingham et al. 2018;
334 Campbell et al. 2019; Vandeputte et al. 2021) and current tagging technologies can be replaced
335 by PBT to provide additional benefits at similar or reduced costs (Beacham 2021). This implies
336 that PBT will continue to grow in usage, and the method described here will become feasible for
337 a larger number of hatchery programs.
338 The simulated microhaplotype panels demonstrated that error rates low enough for
339 relatively large analyses of segregated hatchery programs are achievable with panels containing
340 300 - 400 microhaplotypes. The size/power required from the genetic marker panel will depend
341 on the data available to limit the number of comparisons. As has been previously demonstrated,
342 the availability of accurate hatchery cross-records greatly reduces the power required for
343 grandparent inference (Letcher and King 2001), but we note that simply having sex information
344 associated with a broodstock individual's genotype can have a sizeable effect (Table 1). Panels
345 genotyping hundreds of loci have been created using cost-effective, amplicon sequencing
346 techniques (Campbell et al. 2015; Janowitz-Koch et al. 2019), and so we conclude that
347 grandparent inference with this method is feasible using current genotyping techniques.
348 Application of this method at larger scales will require considering potential grandparents
349 from multiple hatcheries. In these situations, having data available to reduce the number of
350 comparisons can be critical for making an analysis feasible. For example, if natural-origin

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351 steelhead are sampled at Lower Granite Dam on the Snake River (Hargrove et al. 2021), then all
352 steelhead hatcheries adjacent to and upstream of the dam must be considered (approximately
353 5,000 total steelhead spawned annually). With no data other than the year and hatchery at which
354 fish were spawned, the number of potential pairs of grandparents would be approximately $5.5 \cdot$
355 10^6 each year, while considering phenotypic (or genetic) sex would reduce this to $1.4 \cdot 10^6$
356 (IDFG, unpublished).

357 We expect this method to be most applicable for assessing segregated hatchery programs.
358 For some closely related trios, false positive error rates were relatively high with the 300
359 microhaplotype panel (Supplemental Figure 1). For all analyses, the impact of these error rates is
360 mediated by the infrequency of comparisons involving closely related individuals. When
361 analyzing segregated programs, false positives from closely related trios may not negatively
362 impact conclusions as they indicate recent hatchery ancestry. However, when analyzing
363 integrated hatchery programs, distinguishing trios with different relationships can be important.
364 Application of this method could then either be infeasible or require a more powerful genetic
365 panel. Additionally, the number of relationships for which we provide estimators covers trios
366 with a range of relatedness but is not exhaustive. In some specific cases, other relationships may
367 be present at impactful frequencies. For example, if generations overlap substantially then the
368 impact of trios containing aunts, half-aunts, and first-cousins may need to be considered.

369 The method developed here addressed shortcomings of previously developed methods for
370 grandparent inference with moderately sized genetic panels. Previous methods required either
371 that all four grandparents were sampled (Letcher and King 2001) or used an exclusionary method
372 that inherently had lower power than likelihood-based methods (Christie et al. 2011).
373 Additionally, the exclusionary method did not provide a formal treatment of genotyping error.

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374 Applications of the exclusionary method (Christie et al. 2011; Sard et al. 2016) have utilized
375 panels of tens of microsatellites. When using SNPs or microhaplotypes, hundreds or thousands
376 of loci are typically genotyped and minimal error rates (e.g., 1%) still result in errors being
377 present in a majority of individuals. An additional effect of ignoring genotyping error in an
378 exclusionary method is that false negatives are implicitly assumed not to occur. It is worth noting
379 that use of a Markov chain to model the number of observed Mendelian incompatibilities and
380 missing genotypes in a trio, as we developed here, could be incorporated into the exclusionary
381 method. This would allow both the formal incorporation of genotyping error and estimation of
382 false positive and false negative error rates.

383 Importance sampling and stratified sampling have different weaknesses, making each
384 better suited to different situations. For example, importance sampling can perform suboptimally
385 when the estimate is dominated by a small fraction of samples (Owen 2013). Stratified sampling
386 does not have this same drawback, but it can fail to give a meaningful estimate when the false
387 positive rate within a particular stratum is low enough that a reasonable number of samples
388 cannot estimate it accurately. Comparison of the importance sampling and stratified sampling
389 methods showed that when the true relationship was unrelated, they estimated essentially the
390 same false positive rate when that rate was above approximately 10^{-8}. This suggests the
391 importance sampling routine performed well for unrelated trios. At lower false positive rates, the
392 stratified sampling estimates were lower (and in some cases 0) than those from importance
393 sampling. In these cases, one or more strata had estimates of 0, indicating that the 1,000,000
394 samples taken were not sufficient to observe one or more false positives. This demonstrates one
395 of the strengths of importance sampling compared to stratified sampling - some situations will
396 have false positive rates small enough they cannot be efficiently estimated by this stratified

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397 sampling routine. This is further emphasized by the greater computational effort devoted to
398 stratified sampling in this study (1,000,000 iterations in each stratum vs. 1,000,000 iterations
399 total).

400 For false positive error rates under relationships other than unrelated, the importance
401 sampling and stratified sampling estimates were again largely the same. In a few cases where one
402 potential grandparent was the true grandparent and the other was not, the estimates were
403 noticeably different and the stratified sampling method achieved at least 150 false positive
404 observations in each strata. This suggests that for these relationships the importance sampling
405 method may have performed suboptimally. Similar observations were made for importance
406 sampling estimates of false positive error rates in parentage inference, where performance of a
407 given importance distribution varied depending on the true relationship for which an error rate
408 was estimated (Anderson and Garza 2006). One strategy would be to design an alternative
409 importance distribution, but for closely related trios, error rates for most panels will likely be
410 high enough that they are amenable to stratified sampling.

411 The current method assumes loci are in linkage equilibrium. If loci are physically linked
412 (but still in equilibrium) the methods described here can be applied. Physical linkage of two loci
413 will result in grandchildren being more likely to inherit alleles at both loci from one of the two
414 grandparents in a trio. The estimated false positive (when true relationship is unrelated) error
415 rates are not affected because unrelated individuals are not impacted by physical linkage. The
416 Monte Carlo method we implement for estimating false negative error rates simulates trios
417 assuming no linkage, but given the alleles present at two loci in the grandparents, the probability
418 of a given combination of alleles being inherited by the grandchild is identical regardless of
419 physical linkage (Supplemental file 1). Therefore, as long as the set of alleles present in the

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420 grandparents (ignoring which grandparent is assigned which alleles) is simulated accurately, the
421 frequency of combinations of grandparent alleles and grandchild alleles will be simulated
422 accurately by assuming the loci are unlinked. The calculated LLR does not rely on assignment of
423 genotypes to specific grandparents, and so the distribution of LLR, and thus the false negative
424 error rate, is also simulated accurately by assuming the loci are unlinked.

425 One drawback of the method as implemented is that computational efficiency decreases
426 with increasing numbers of alleles per locus. This is partly due to the flexibility of the
427 genotyping error model and the need to marginalize over all possible true genotypes. In the
428 current implementation, computation is sped up by precomputing likelihood values for observed
429 trio genotypes at each locus. This works well when the number of alleles per locus is small, but
430 as the number of alleles increases, this can become impractically slow. As such, the current
431 implementation will be most suitable to panels containing biallelic SNPs and microhaplotypes,
432 which in our experience typically have three to five alleles. For panels of highly variable loci, a
433 different implementation of this method, particularly with a more streamlined genotyping error
434 model, would be necessary.

435

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440

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571

572

Hatchery	Data used	Number of possible crosses per year	Years of potential grandparents	Number of potential grandchildren	Number of comparisons	Desired per-comparison false positive error rate
USB	None	4,290			3,432,000	$2.91 \cdot 10^{-8}$
	Sex	1,088			870,400	$1.15 \cdot 10^{-7}$
	Spawn day	584			467,200	$2.14 \cdot 10^{-7}$
	Spawn day and sex	162			129,600	$7.72 \cdot 10^{-7}$
	Cross records	34	4	200	27,200	$3.68 \cdot 10^{-6}$
DNFH	None	3,134,670			2,507,736,000	$3.99 \cdot 10^{-11}$
	Sex	769,348			615,478,400	$1.62 \cdot 10^{-10}$
	Spawn day	284,270			227,416,000	$4.40 \cdot 10^{-10}$
	Spawn day and sex	69,709			55,767,200	$1.79 \cdot 10^{-9}$
	Cross records	1,003			802,400	$1.25 \cdot 10^{-7}$

573 Table 1. Desired per-comparison false positive (true relationship of unrelated) error rates for analyses using different data sources to
574 reduce the number of comparisons.

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575 Figure Legends

576 Figure 1. The pedigree the proposed method could be used to infer. H: hatchery-origin, W:
577 natural-origin

578 Figure 2. Error rates estimated by importance sampling for simulated (A) SNP and (B)
579 microhaplotype panels with varying numbers of loci.

580 Figure 3. Comparison of false positive (true relationship of unrelated) error rates for simulated
581 microhaplotype panels estimated by importance sampling and stratified sampling. The black line
582 represents y = x. Points shown on the y-axis had an estimated error rate of 0 from stratified
583 sampling.

584 Figure 4. Comparison of false positive error rates estimated by importance sampling and
585 stratified sampling for related trios with the simulated 300 locus microhaplotype panel. The
586 black line represents y = x. The two putative grandparents were unrelated to each other. The
587 relationship labels indicate the relationship of the two putative grandparents to the putative
588 grandchild. Where the label is only one relationship, both putative grandparents have the same
589 relationship to the putative grandchild. True is true grandparent, GAunt is great-aunt, HGAunt is
590 half great-aunt, GpCous is first-cousin to the putative grandchild's true grandparent, Unrel is
591 unrelated. The inset gives a magnified view of the region containing error rates close to 1.

592 Figure 5. Error rates estimated by importance sampling for the simulated 300 locus
593 microhaplotype panel with two rates of missing genotypes: 3% (Missing) and 0% (No missing).
594 False positive error rate is the rate for unrelated trios.

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595 Supplemental Figure 1. Error rates estimated by stratified sampling for the simulated 300 locus
596 microhaplotype panel for select related trios. The two putative grandparents were unrelated to
597 each other. The relationship labels indicate the relationship of the two putative grandparents to
598 the putative grandchild. True is true grandparent, GAunt is great-aunt, HGAunt is half great-aunt,
599 Unrel is unrelated.

600

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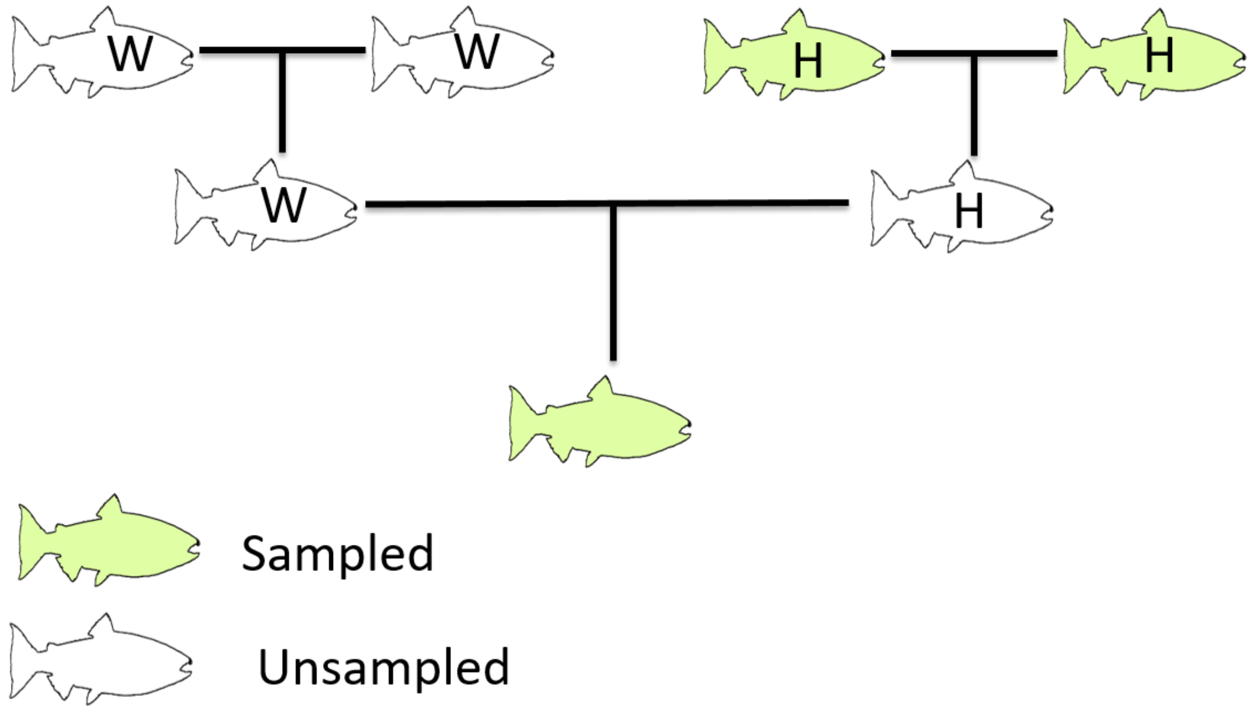


A

False positive

1e−02
1e−06
1e−10
1e−14

0.00 0.01 0.02 0.03 0.04 0.05

False negative

Number of loci

100
300
500
700
900

B

False positive

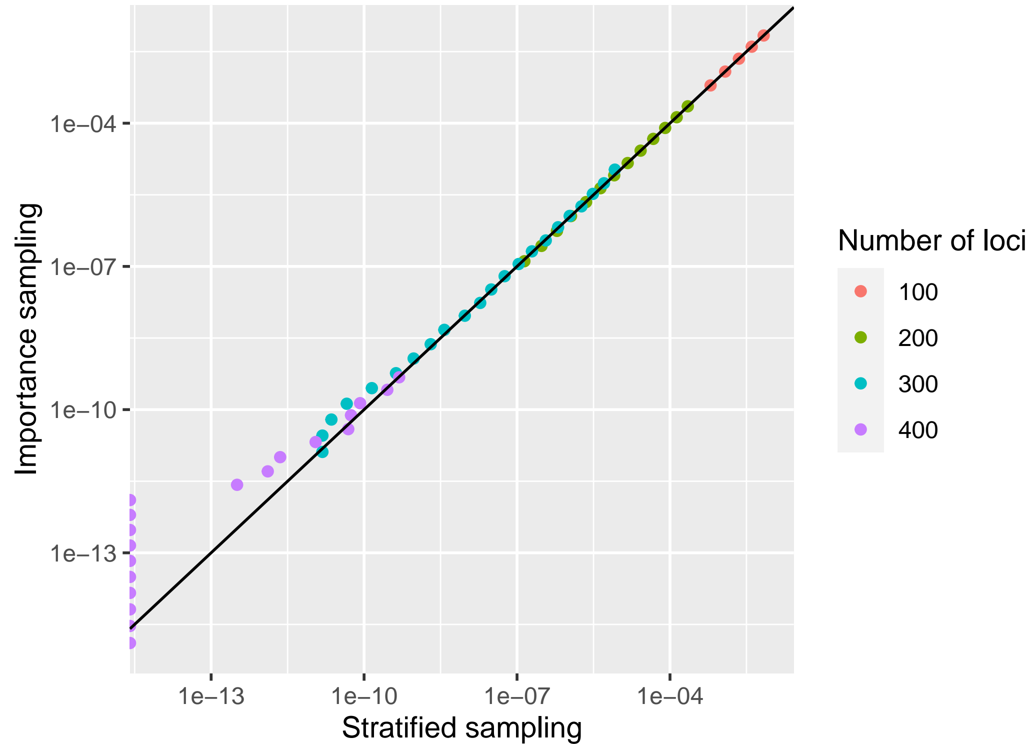
1e−02
1e−06
1e−10
1e−14

0.00 0.01 0.02 0.03 0.04 0.05

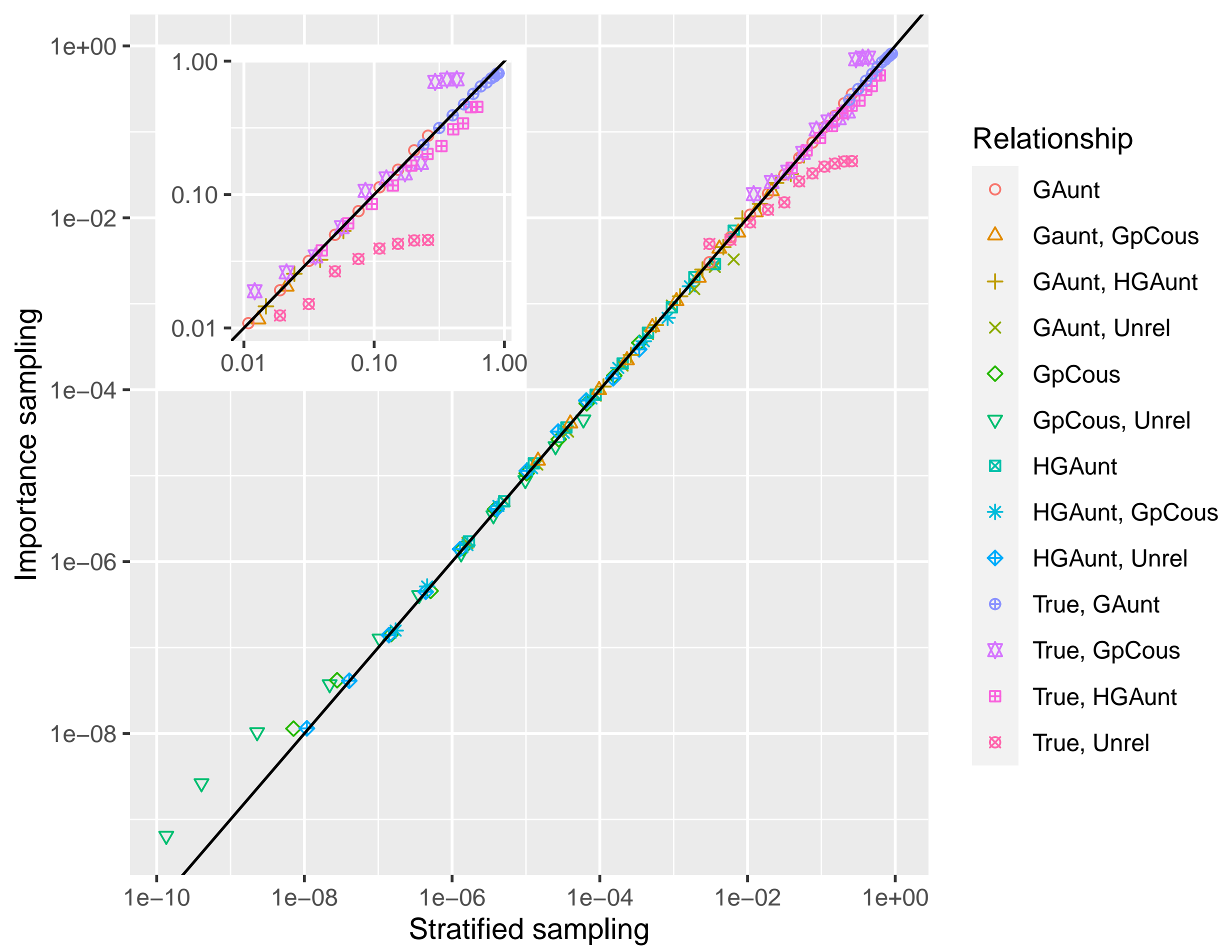
False negative

Number of loci

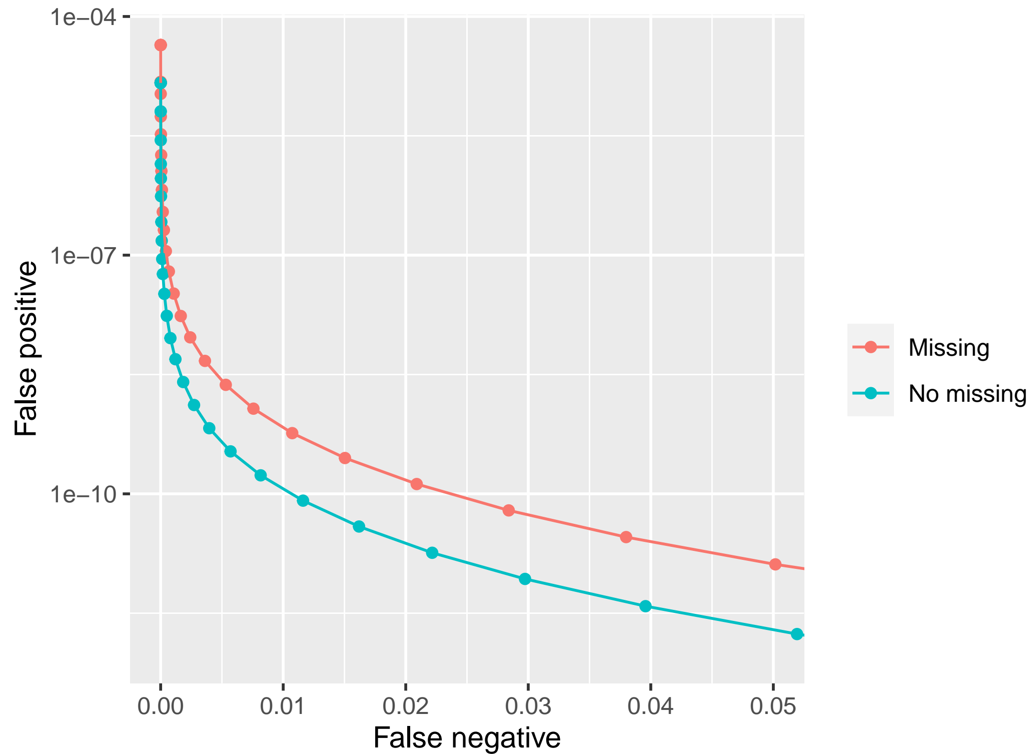
100
200
300
400

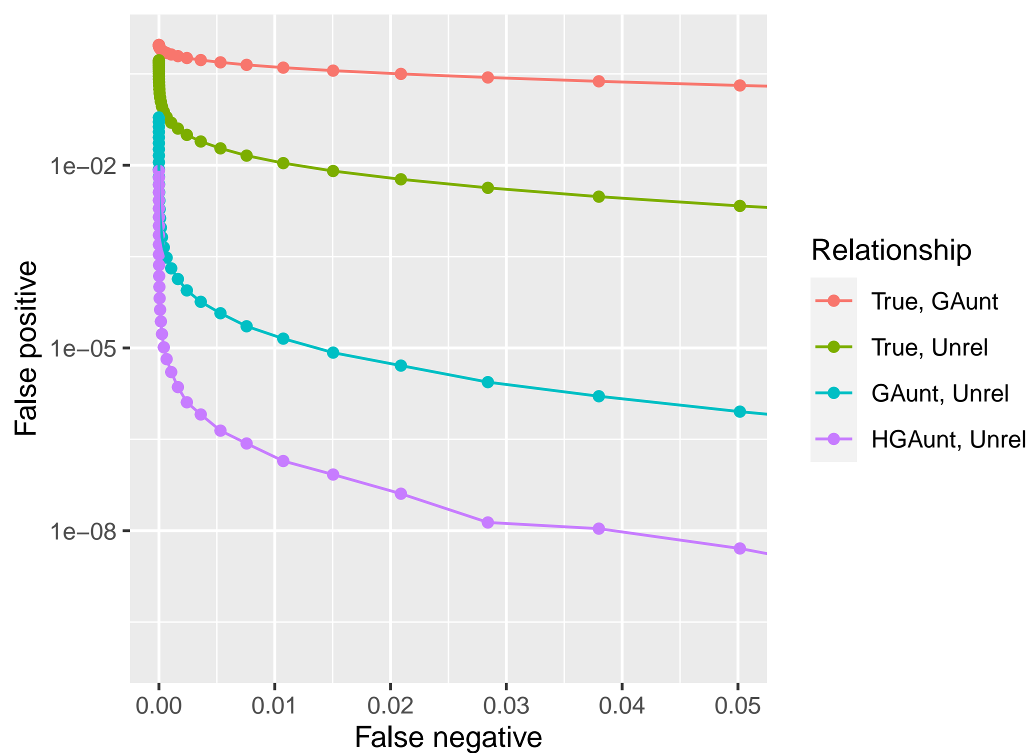


Importance sampling
Stratified sampling
1e−04
1e−07
1e−10
1e−13
1e−13
1e−10
1e−07
1e−04
Number of loci
100
200
300
400



Importance sampling
Stratified sampling
1e+00
1e−02
1e−04
1e−06
1e−08
1e−10
1.00
0.10
0.01
Relationship
GAunt
Gaunt, GpCous
GAunt, HGAunt
GAunt, Unrel
GpCous
GpCous, Unrel
HGAunt
HGAunt, GpCous
HGAunt, Unrel
True, GAunt
True, GpCous
True, HGAunt
True, Unrel


1e−04
1e−07
1e−10
False positive
0.00
0.01
0.02
0.03
0.04
0.05
False negative
Missing
No missing



False positive
1e−02
1e−05
1e−08
0.00
0.01
0.02
0.03
0.04
0.05
False negative
Relationship
True, GAunt
True, Unrel
GAunt, Unrel
HGAunt, Unrel

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Supplementary file 1

1 Default error model for gRandma

Each locus is treated separately and each genotype is treated as two observations (one for each allele in the presumed diploid). For a given locus, the error model first relies upon a "per allele error rate", ϵ, which represent the probability that, when observing one allele, you observe any allele other than the true one. The default value is 0.005, or 0.5%. This value is used along with a measure of similarity between alleles to calculate the probability of observing each allele given the true allele. For a locus with I alleles, given the true allele i, the probability of correctly observing i is $1 - \epsilon$ and the probability of observing allele j where $j \neq i$ is

$$P(j|i) = \epsilon \cdot \frac{s_{ij}}{\sum_{k=1}^{I} s_{ik}}, \quad (1)$$

where s_{ij} is the similarity between alleles i and j and $s_{ii} = 0$. The measure of similarity between two alleles of a microhaplotype used here was the reciprocal of the number of base pair differences between them.

These probabilities are then used to calculate the probability of observing each genotype given a true homozygous genotype. The probability of observing a genotype of BC given a true genotype of AA is

$$P(BC|AA) = \begin{cases} 2 \cdot P(B|A)P(C|A), & B \neq C \\ P(B|A)^2, & B = C, \end{cases} \quad (2)$$

according to basic probability arguments, where $P(B|A)$ is the probability of observing B when the true allele is A. The probability of observing each possible genotype given a true heterozygous genotype is calculated similarly, but also incorporates a probability of allelic dropout for each allele. Each allele is given a probability of dropping out, d_i for allele i, and in the current study a probability of 0.005 was used for all alleles. It is assumed that allelic dropout events are disjoint, and so the probability of no dropout occurring is $1 - d_i - d_j$. The probability of observing each genotype given no dropout can be calculated using the observation probabilities for each allele calculated in equation 1. The probability of observing a given genotype if a dropout has occurred is taken to be the probability of observing that genotype given a true homozygous genotype for the remaining allele. The total probability of observing a genotype is the sum of

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24 the probabilities of all three cases (no dropout, allele 1 dropout, allele 2 dropout).

25 2 Physical linkage

26 In the combined genotypes of the grandparents, at locus 1 alleles A_1, A_2, A_3, A_4 are present, and at locus 2
27 alleles B_1, B_2, B_3, B_4 are present. If the loci are unlinked, the probability of the grandchild inheriting A_1, B_1
28 from the grandparents is $0.25 \cdot 0.25 = 0.0625$. If the loci are linked, there are three cases to consider:

29 1. A_1 and B_1 are on the same chromosome

30 2. A_1 and B_1 are on different chromosomes in the same grandparent

31 3. A_1 and B_1 are on different chromosomes in different grandparents

32 The probability of case 1 is 0.25, because the loci are in linkage equilibrium, and so B_1 is equally likely to
33 be on the same chromosome as any of the alleles at locus 1. Similarly, the probability of cases 2 and 3 are
34 0.25 and 0.5, respectively.

35 Under case 1, the probability the unsampled parent inherits A_1 and B_1 is $0.5(1 - r)$, where r is the
36 probability of a recombination event and the probability the grandchild then inherits A_1 and B_1 is $0.5(1-r)$.
37 So, the probability the grandchild inherits A_1 and B_1 under case 1 is $0.25(1 - r)^2$.

38 Under case 2, the probability the unsampled parent inherits A_1 and B_1 is $0.5r$ and the probability the
39 grandchild then inherits A_1 and B_1 is $0.5(1-r)$. So, the probability the grandchild inherits A_1 and B_1 under
40 case 2 is $0.25r(1 - r)$.

41 Under case 3, the probability the unsampled parent inherits A_1 and B_1 is $0.5 \cdot 0.5 = 0.25$ and the
42 probability the grandchild then inherits A_1 and B_1 is $0.5r$. So, the probability the grandchild inherits A_1
43 and B_1 under case 3 is $0.125r$.

44 Combining all three cases, we find that the probability of a grandchild inheriting A_1 and B_1 from the
45 grandparents is

$$0.25 \cdot 0.25(1 - r)^2 + 0.25 \cdot 0.25r(1 - r) + 0.5 \cdot 0.125r \tag{3}$$

$$0.0625(1 - r)^2 + 0.0625r(1 - r) + 0.0625r \tag{4}$$

$$0.0625((1 - r)^2 + r(1 - r) + r) \tag{5}$$

$$0.0625(1). \tag{6}$$