Double-digest RAD sequencing outperforms microsatellite loci at assigning paternity and estimating relatedness: a proof of concept in a highly promiscuous bird

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Abstract

Information on genetic relationships among individuals is essential to many studies of the behavior and ecology of wild organisms. Parentage and relatedness assays based on large numbers of SNP loci hold substantial advantages over the microsatellite markers traditionally used for these purposes. We present a double-digest restriction site-associated DNA sequencing (ddRAD-seq) analysis pipeline that, as such, simultaneously achieves the SNP discovery and genotyping steps and which is optimized to return a statistically powerful set of SNP markers (typically 150-600 after stringent filtering) from large numbers of individuals (up to 240 per run). We explore the tradeoffs inherent in this approach through a set of experiments in a species with a complex social system, the variegated fairy-wren (*Malurus lamberti*), and further validate it in a phylogenetically broad set of other bird species. Through direct comparisons with a parallel dataset from a robust panel of highly variable microsatellite markers, we show that this ddRAD-seq approach results in substantially improved power to discriminate among potential relatives and considerably more precise estimates of relatedness coefficients. The pipeline is designed to be universally applicable to all bird species (and with minor modifications to many other taxa), to be cost- and time-efficient, and to be replicable across independent runs such that genotype data from different study periods can be combined and analyzed as field samples are accumulated.

Introduction

Advances in molecular techniques over the past several decades have substantially improved our ability to test questions about animal social behavior by providing reliable information on the genetic relationships among individuals (Westneat *et al.* 1990; Hughes 1998; Avise *et al.* 2002; Griffith *et al.* 2002; Solomon *et al.* 2004; Myers & Zamudio 2004). Microsatellites have been the molecular 'tool-of-choice' for this application since the 1990s, as microsatellite loci are often highly polymorphic, with up to dozens of co-segregating alleles at a single locus (Queller *et al.* 1993; Li *et al.* 2002; Selkoe & Toonen 2006; Guichoux *et al.* 2011). Accordingly, a small number of highly variable microsatellite loci can provide considerable power for discerning genetic relationships among individuals (Queller *et al.* 1993; Blouin 2003; Webster & Reichart 2005). However, microsatellite assays also have some practical drawbacks. Microsatellite laboratory protocols developed for one species are often not suitable for use in other species because the primers may not amplify well and targeted loci are often not as polymorphic, especially in more distantly related taxa (Galbusera 2000; Decroocq *et al.* 2003; Hedgecock *et al.* 2004; Primmer *et al.* 2005). Next-generation sequencing has made the discovery of microsatellite loci for individual species more attainable (Davey *et al.* 2011). However, discovering microsatellite loci can be very time consuming and costly, largely due to protracted testing and optimization of candidate primers after the initial sequencing. Additionally, traditional PCR-based microsatellite assays also incur substantial financial and lab-bench time investments. The manual scoring of microsatellite alleles also requires substantial researcher time, and can

88 involve various forms of error arising from alleles that have more than one clearly
89 defined peak, allelic drop-out and null allele issues, and the various sources of human
90 error that are inherent in any complicated workflow (Pemberton *et al.* 1995; Hedgecock
91 *et al.* 2004; Hoffman & Amos 2005; Kalinowski *et al.* 2007).
92 Many of these limitations are less severe in assays based on single-nucleotide
93 polymorphisms (SNPs), which require fewer steps and have greater automation (Gut
94 2001; Syvänen 2001; Seeb *et al.* 2011; Davey *et al.* 2011). SNPs are appropriate
95 alternatives for studies of parentage and relatedness data because they are abundant in
96 the genome, have low mutation rates (Brumfield *et al.* 2003; Morin *et al.* 2004), and can
97 be scored semi-automatically (Garvin *et al.* 2010; Guichoux *et al.* 2011). In comparison
98 to microsatellite-based relationship tests, the primary limitation of SNPs is that they are
99 typically biallelic, whereas microsatellite loci are often multiallelic, and hence the
100 statistical power of SNP loci for discriminating parentage and relatedness is far lower on
101 a per-locus basis (Ball *et al.* 2010). Compared to highly variable microsatellite loci, a
102 substantially higher number of SNP markers is therefore required to achieve appropriate
103 power in parentage and relatedness studies (Glaubitz *et al.* 2003; Morin *et al.* 2004;
104 Coates *et al.* 2009).
105 Recently, the application of SNPs for use in analyses of parentage, relatedness,
106 and overall population structure has received greater attention (Glaubitz *et al.* 2003;
107 Anderson & Garza 2006; Coates *et al.* 2009). Studies in birds (Cramer *et al.* 2011;
108 Weinman *et al.* 2015; Kaiser *et al.* 2017), fish (Hauser *et al.* 2011), and several
109 domesticated taxa (Tokarska *et al.* 2009; Fernández *et al.* 2013) have developed

110 sufficiently large SNP panels to attain a comparable, if not better, level of resolving
111 power as highly polymorphic microsatellite panels. While each of these studies manage
112 to identify powerful SNP panels, the SNP genotyping methods used are often labor
113 intensive, requiring a significant amount of preparatory work at the discovery stage prior
114 to genotyping of large numbers of individuals. Many of these methods also rely on
115 reference genomes (Anderson & Garza 2006; Heylar *et al.* 2011), or other genomic
116 resources (Fernández *et al.* 2013; Weinman *et al.* 2015; Kaiser *et al.* 2017) (e.g.
117 transcriptome, SNP microarray), for SNP identification. Ultimately, this has afforded
118 several beneficial examples of the utility of SNPs for parentage and relatedness
119 analyses, but without an efficient, universal method of SNP discovery and identification.
120 Restriction site-associated DNA sequencing (RAD-seq) is a reduced-
121 representation genomic technique that is widely used in molecular genetic studies
122 (Davey & Blaxter 2010; Etter *et al.* 2012; Puritz *et al.* 2014), particularly for linkage and
123 quantitative trait locus (QTL) mapping (Baird *et al.* 2008), genome wide association
124 studies (Davey *et al.* 2011), and phylogeography (Andrews *et al.* 2016). RAD-seq uses a
125 restriction enzyme to fragment and sample a fraction of a genome; as it identifies SNPs
126 with no prior knowledge of the genome, it provides a more universal method of SNP
127 discovery (Willing *et al.* 2011). Double-digest restriction site-associated DNA sequencing
128 (ddRAD-seq) allows for selection of an even smaller fraction of the genome through the
129 combined use of two restriction enzymes and a subsequent size selection step, affording
130 the ability to target a smaller total number of SNPs in a greater number of individuals
131 (Peterson *et al.* 2012; Puritz *et al.* 2014; Kess *et al.* 2016). This ability, in concert with

132 the fact that no prior knowledge of the genome is needed, makes ddRAD-seq an
133 attractive method of simultaneous SNP discovery and screening for use in discerning
134 genetic relationships among individuals.

135 Here, we describe a ddRAD-based approach to the simultaneous discovery and
136 screening of high numbers of SNP loci with high power for testing questions about
137 parentage and relatedness. These protocols are optimized to generate an appropriately
138 robust set of SNP markers for 240 individuals per run, to be repeatable across runs to
139 allow the combination of SNP datasets generated at different times, and to be
140 universally applicable to birds (and with small modifications, to other organisms) without
141 requiring a species-specific marker discovery step. We validate these methods by
142 conducting a SNP-based parentage and relatedness study in the highly promiscuous,
143 and socially complex, variegated fairy-wren (*Malurus lamberti*). We compare the results
144 with previously generated paternity assignments and relatedness information, based on
145 microsatellite screens of the same fairy-wren individuals and social groups. To illustrate
146 the broad utility of this method we report the number of loci recovered for equivalent
147 studies of parentage that included different numbers of individuals (from less than 10 to
148 almost 500) of a variety of other species that collectively span much of the phylogenetic
149 diversity of living birds.

150

151 **Methods & Materials**

152 *Study population*

153 The variegated fairy-wren, endemic to Australia, is a cooperatively breeding bird
154 that lives in social groups composed of kin and non-kin (Schodde 1982; Rowley &
155 Russell 1997). Male dispersal is limited, and rates of extra-pair fertilizations (EPFs) are
156 high (~68% of all young, assessed with a panel of 12 species-specific microsatellites; DJ
157 Thrasher, unpublished data). We intensively monitored a color-banded population of the
158 nominate subspecies, *M. l. lamberti*, on Lake Samsonvale (27°16' S, 152° 41' E), 30 km
159 northwest of Brisbane, Queensland, Australia, from 2012 – 2016. The population
160 ranges from about 250-300 adults depending on year-to-year conditions. The study site
161 is bounded on most sides by Lake Samsonvale, and on its westernmost side by a major
162 highway, which increases our confidence in sampling most, if not all, of the adults in the
163 population. We also monitored all nesting attempts to measure, mark, and collect blood
164 samples from nestlings 6 days after hatching. Blood samples were immediately stored in
165 lysis buffer (White & Densmore 1992), and genomic DNA was later extracted using
166 Qiagen DNeasy Blood and Tissue kits. DNA concentration was determined using the
167 Qubit dsDNA BR Assay Kit and the Qubit® Fluorometer (Life Technologies) following
168 the manufacturers protocol.
169
170 *Microsatellite development and genotyping*
171 We previously developed twelve polymorphic microsatellite loci for the variegated
172 fairy-wren (Table S1, Supporting Information) following methods described previously in
173 Nali *et al.* (2014). Briefly, we extracted genomic DNA from blood in lysis buffer from eight
174 adults in our study population, enriched the mix of DNA with repetitive sequences to

175 develop an enriched microsatellite library, and conducted an Illumina MiSeq sequencing
176 run. From this pool of sequences, we optimized twelve loci that amplified well using
177 polymerase chain reaction (PCR), were polymorphic, and exhibited clearly defined
178 peaks for genotyping. We designed three multiplexed PCRs for genotyping, and each
179 amplification reaction contained 1ul of genomic DNA of varying concentrations (1 ng/ul -
180 40 ng/ul). PCR products were combined with the GeneScan 500 base pair LIZ internal
181 size standard for size-sorting using a 3730 DNA Analyzer. We used Geneious version
182 8.0 (Kearse *et al.* 2012) to score alleles. The program automatically identifies alleles at
183 each locus, and we manually inspected allele calls to minimize genotyping error. In total,
184 we genotyped 287 adults and 482 nestlings from 226 nests sampled during the 2012-
185 2016 breeding seasons.
186
187 *ddRAD sequencing*
188 We selected a subset of the individuals genotyped for microsatellite loci (120
189 adults and 40 nestlings) for use in our ddRAD-seq experiment and subsequent
190 analyses. To assess the reliability of our SNP panel for parentage and relatedness
191 analysis, we chose representative nestlings from all the years of our study. Typically, we
192 selected one nestling from any individual nest. In a few cases, we selected two nestlings
193 that prior microsatellite analysis had assigned to the same mother but different fathers.
194 For each nestling, we included the mother, the social father, and the genetic father as
195 assigned by previous microsatellite analysis. Our pool of candidate parents included 24

196 mothers and 78 putative fathers, and 18 randomly selected individuals of both sexes, for
197 a total of 120 adults.

198 Our ddRAD-seq protocol is adapted from Peterson et al. (2012) (see Supporting
199 Information for a detailed protocol). Briefly, for each individual, 100ng - 500ng of DNA
200 (20ul of DNA between concentrations of 5ng/ul - 25ng/ul) were digested with either SbfI
201 and MspI, or SbfI and EcoRI (NEB), and ligated with one of 20 P1 adapters (each
202 containing a unique inline barcode) and a P2 adapter (P2-MspI or P2-EcoRI). After
203 digestion and ligation these samples were pooled in groups of 20 (each with a unqiue P1
204 adpater) and purified using 1.5X volumes of homemade MagNA made with Sera-Mag
205 Magnetic Speed-beads (FisherSci) as described by Rohland & Reich (2012). Fragments
206 of between 450 bp and 600 bp were selected using BluePippin (Sage Science) by the
207 Cornell University Biotechnology Resource Center (BRC). Following size selection,
208 index groups and Illumina sequencing adapters were added by performing 11 PCR
209 cycles with Phusion DNA Polymerase (NEB). These reactions were cleaned up with 0.7x
210 volumes of MagNA and pooled in equimolar ratios to create a single library for
211 sequencing on one lane of Illumina HiSeq 2500 (100 bp single end, performed by BRC).
212 The sequencing was performed with a ~10% PhiX spike-in to introduce diversity to the
213 library.

214 By replicating 20 samples (run as a separate index group) with a wider
215 BluePippin size selection range of 400-700 bp, we explored the inherent trade-off
216 between the number of samples sequenced on a lane at a given coverage threshold and
217 the number of SNPs recovered per sample. We similarly explored the effects of using a

218 less frequent restriction enzyme digest (by substituting the 6 bp cutter EcoRI in place of
219 the 4 bp cutter MspI) to generate a smaller total number of fragments in our size range,
220 which in turn should increase the sequencing coverage of the loci screened.

221 To assess repeatability across index groups, we replicated two index groups,
222 each comprised of 20 samples: one index group from the standard protocol, and the
223 index group generated with the rare-cutter EcoRI enzyme (Table 1). We multiplexed the
224 resulting 240 samples in 12 index groups (with 20 individuals each) which were pooled
225 into a final library that was sequenced on one lane of an Illumina HiSeq 2500, producing
226 220,300,739 100 bp single end reads. Eight index groups included the samples used for
227 assigning paternity and estimating relatedness, and four additional index groups were
228 included to assess the variation in the number of loci recovered with changes in our
229 molecular protocol (choice of enzyme and size selection window), and to assess
230 repeatability (see Table 1). To avoid an additional source of variation these last four
231 index groups each included the same 20 individuals.

232

233 *SNP data analysis*

234

235 <u>Quality filtering and demultiplexing</u>

236 After the quality of the reads was assessed using FASTQC version 0.11.5
237 (www.bioinformatics.babraham.ac.uk/projects/fastqc), we trimmed all sequences to 97bp
238 using fastX_trimmer (FASTX-Toolkit) to exclude low quality calls near the 3' of the reads.
239 We subsequently removed reads containing at least a single base with a Phred quality

240 score of less than 10 (using fastq_quality_filter). We additionally removed sequences if
241 more than 5% of the bases had a Phred quality score of less than 20. Using
242 process_radtags module from the Stacks version 1.37 pipeline (Catchen *et al.* 2013), we
243 demultiplexed the reads to obtain files with sequences that were specific to each
244 individual.
245
246 <u>De novo assembly of RAD loci</u>
247 Because we do not have a sequenced genome for the variegated fairy wren or a
248 close relative – which is likely to be the case for many non-model organisms involved in
249 parentage studies – we assembled the sequences de novo using the Stacks pipeline
250 (Catchen *et al.* 2013). If the genome of the species of interest (or a closely related
251 species) is available, a reference based assembly of RAD loci is preferred (Shafer et al.
252 2017). First, we used denovo_map.pl to assemble the reads into a catalog allowing a
253 minimum stack depth of 5 (m parameter), up to 5 mismatches per locus within an
254 individual (M paramenter), and 5 mismatches between loci of different individuals when
255 building the catalog (n parameter). This combination of parameters has been shown to
256 work well for other similarly polymorphic passerine birds (Campagna *et al.* 2015), but we
257 expect the optimal set of parameters to vary across datasets. For a detailed exploration
258 on how the different assembly parameters in stacks impact the number and quality of
259 loci recovered, see Paris *et al.* (2017). We ran the rxstacks module to filter loci with a log
260 likelihood of less than -50 (lnl_lim -50) or that were confounded in at least 25% of the

261 population (conf_lim 0.25). We then built a new catalog by rerunning cstacks and
262 obtained individual genotype calls with sstacks.
263
264 SNP filtering
265 SNPs were exported using the populations module of the Stacks pipeline. All of
266 our samples were grouped in one population and a locus was exported if it was present
267 in 95% of the individuals in this population (r parameter) at a stack depth of at least 10
268 (m parameter). When a RAD locus had more than one SNP, the data were restricted to
269 the first one (--write_single_snp) to avoid including SNPs in high linkage disequilibrium
270 (LD). We required a minor allele frequency of at least 0.25 to process a nucleotide site (-
271 -min_maf).
272 Using vcftools version 0.1.14 (Danecek *et al.* 2011), we removed loci that were
273 not in Hardy-Weinberg equilibrium (---hwe). We also confirmed that LD was low by
274 calculating r2 values for every pairwise combination of SNPs in the final dataset using
275 vcftools (data not shown). We obtained a variant call format (vcf) file that was converted
276 to genepop format in PGD Spider version 2.0.5.0 (Lischer & Excoffier 2012) and
277 imported into CERVUS version 3.0.7 (Kalinowski *et al.* 2007).
278
279 Assessing repeatability and overlap in different sets of RAD loci
280 We conducted five independent de novo assemblies using stacks, one for our
281 larger set of 160 samples, and one for each of the four index groups included in the
282 sequencing run to assess repeatability and understand the impact of variations in the

283 protocol on the number of loci recovered (Table 1). Once the assemblies were
284 completed, we filtered and exported loci independently as described above. We then
285 queried what the overlap among these sets of loci was (e.g., between the full set of 160
286 samples and a replicate of 20 samples – index 9). To better understand sources of
287 variation in our experimental protocol we also asked if the loci retained after filtering
288 were present in the catalogs of different assemblies. To assess overlap in sets of RAD
289 loci we first generated FASTA files with the sequence data from both the filtered loci
290 from each assembly and from the entire catalog. We then used blast version 2.3.0
291 (Altschul *et al.* 1990) with an E-value of 1e-10 to find matches among these FASTA files
292 and calculate the proportion of RAD tags shared by the different sets of sequences
293 (Tables 2 and 3).
294
295 *Parentage analysis*
296 We used CERVUS version 3.0.7 (Kalinowski *et al.* 2007) to assign paternity for all
297 nestlings using our microsatellite and SNP datasets separately. CERVUS uses a two-
298 step, likelihood-based approach to assign parentage. First, CERVUS compares each
299 offspring's genotype to that of a candidate parent and a random individual in the
300 population to calculate a likelihood ratio. This relationship is presented as an LOD score,
301 which is simply the natural logarithm of the calculated likelihood ratio. Positive LOD
302 scores indicate that a candidate parent is much more likely to be the true parent,
303 whereas negative LOD scores indicate that the candidate parent is highly unlikely to be
304 a true parent. Second, CERVUS conducts a simulation of parentage analysis based on

305 population allele frequencies and the proportion of potential parents included in the
306 analysis. The simulation accounts for the possibility of unsampled parents, missing data,
307 and genotyping errors. Considering these parameters, the simulation calculates critical
308 LOD scores by comparing the LOD distributions of the most likely parent and all other
309 candidate parents. The critical LOD score is used to determine the confidence (95% or
310 80%) of each parentage assignment.

311 CERVUS allows for different types of parentage analysis, including parent-pair
312 (sexes known or unknown), maternity (known father, but not mother), and paternity
313 (known mother, but not father). Variegated fairy-wrens at Lake Samsonvale are relatively
314 easy to observe, and we were able to assign known mothers behaviorally. We
315 subsequently confirmed this with microsatellite analyses: females that built and attended
316 a nest throughout incubation were always the mothers of the nestlings in that nest. In
317 many systems, a comparable level of demographic knowledge may not be available, so
318 a marker set must be powerful enough to assign parentage with minimal social
319 information. To investigate the broader utility of our ddRAD-seq method, we conducted
320 analyses that relied on the inclusion of the known mother, in addition to analyses
321 independent of the known mother, which were based only on the father-offspring
322 relationship. We simulated paternity assignments for 10,000 offspring to determine
323 critical LOD scores, using slightly different input parameters for each panel
324 (microsatellites and ddRAD sequencing derived SNPs). Simulations for both used the
325 following parameters: 78 candidate males, 95% of candidate males sampled, estimated
326 error rate of 0.01 for mistyped loci and likelihood scores. The proportion of loci typed

327 across all individuals was different for both panels: 0.997 for the microsatellite
328 simulation, and 0.961 for the SNP simulation.
329 For both paternity analyses, we used the trio LOD score and the father-offspring
330 LOD score from CERVUS to make assignments. The trio LOD score was calculated by
331 comparing the genotypes of the candidate male and offspring, relative to that of the
332 known mother. The father-offspring LOD score only accounts for the relationship of the
333 candidate male and the offspring, independent of the known mother. CERVUS ranked
334 candidate males by LOD scores in each category, and the highest-ranking males were
335 assigned as fathers. These rankings should be in agreement, but ambiguous
336 assignments (different top-ranking males assigned in each category) may occur when
337 multiple candidate male genotypes closely match an offspring's genotype.
338 We assessed each CERVUS assignment to determine whether it was plausible,
339 and whether the assigned male was the social father or an extra-pair sire. Our criteria for
340 accepting assignments differed slightly for microsatellites and SNPs. For microsatellites,
341 we automatically accepted the CERVUS assignment if the highest-ranking male was in
342 agreement for both the trio LOD and the father-offspring LOD, and if the number of
343 mismatches between the assigned male and the offspring was ≤ 1 (8% of 12 loci). For
344 SNPs, we also accepted the assignment if the highest-ranking males by LOD score type
345 were in agreement, and an allowable number of mismatches were not exceeded.
346 However, for SNPs, our allowable number of mismatches was based on the observed
347 maximum number of mismatches between a known mother and her known offspring
348 (max. = 7, mean = 3.4, 2% of 411 loci). For both panels, we accepted the social father

349 as the genetic sire if he met these respective criteria. If the social father mismatched the
350 offspring at higher numbers, or had negative LOD scores, the offspring was considered
351 sired by an extra-pair father. We accepted assignments of extra-pair fathers using the
352 same criteria outlined above. We did not observe cases in which an offspring could not
353 be assigned to either its social father, or an extra-pair sire.
354
355 *Relatedness analysis*
356 We used the package, 'related' (Pew *et al.* 2015), in R version 3.2.5 (R Core
357 Team 2016) to estimate pairwise relatedness (r) between all pairs of individuals in this
358 study. This package accounts for genotyping errors, missing data, and can estimate
359 relatedness using any of seven different estimators (4 non-likelihood-based, and 3
360 likelihood-based). 'Related' includes the function, **compareestimators**, which tests the
361 performance of different estimators on simulated data that share the same
362 characteristics as the real data. The program uses an allele frequency file to generate
363 simulated pairs of individuals of known relatedness, and automatically estimates
364 relatedness using four of the most commonly used estimators (all non-likelihood-based).
365 The function calculates a correlation coefficient between observed and expected values,
366 to evaluate which estimator performs best with the data set. Using **compareestimators**
367 to generate 200 simulated pairs of individuals for each degree of relatedness (i.e., half-
368 sib, full-sib, parent-offspring, unrelated), we determined that the Wang (2002) estimator
369 performed best for both our microsatellite and SNP datasets. We obtained point

370 estimates of relatedness using the Wang (2002) estimator, and evaluated all parent-
371 offspring relationships that were previously determined in our parentage analysis.
372 'Related' also evaluates how well different marker sets resolve degrees of
373 relatedness, given simulated genotypes based on allele frequency files. For both panels,
374 we used the **familysim** function to generate 200 pairs of individuals for each degree of
375 relatedness. We then used the **coancestry** function to analyze all pairwise relatedness
376 values with the Wang (2002) estimator. We created density plots representing
377 histograms of the relatedness values. These plots show the overlap in relatedness
378 values for degree of relatedness, and we used them to infer how well each panel
379 performed at discerning different relationships.
380
381 *Comparison with other avian ddRAD datasets*
382 We applied the same molecular protocol and bioinformatics pipeline described
383 above to other avian species, with the objective of assigning paternity and estimating
384 relatedness. These datasets ranged between 6 and 480 samples, and we calculated the
385 number of loci recovered for comparisons with the current data and to assess the utility
386 of our method with larger sample sizes. We also genotyped an additional 213 variegated
387 fairy-wren individuals and re-analyzed the data in combination with the 160 samples
388 included in this study.
389
390 **Results**
391 *SNP development and analysis*

392 After trimming, filtering and demultiplexing the data, we retained a total of
393 109,524,874 reads across all index groups, with an average of approximately 9,000,000
394 reads per index group. Two individuals failed (i.e., had less than 66,000 reads each; one
395 individual from each of two index groups) and were excluded from further analysis. The
396 number of reads per sample for the remaining individuals ranged from 213,544 to
397 810,966 (mean = 459,726 ± 118,771 std. dev.).
398 Further analysis using the population program from stacks identified loci with at
399 least 10X coverage, present in 95% of the individuals, and a minimum allele frequency
400 greater than 25%. We further retained only those loci that were in Hardy-Weinberg
401 equilibrium. When performing analyses on all 160 individuals from the primary
402 comparison runs (Table 1), we identified 411 loci that fulfilled these criteria and were
403 used for downstream analyses.
404 We varied two aspects of the ddRAD-seq protocol to assess the number of loci
405 recovered and the reproducibility of the method. As expected, both a reduction in the
406 range of fragment sizes selected during the construction of the library (compare groups
407 A (150 bp size selection) and C (300 bp size selection)), or the use of the less frequent
408 cutter, EcoRI (groups D and E), resulted in fewer loci recovered (Table 2). Those from
409 the EcoRI digest were mostly non-overlapping with those from the MspI index groups.
410 More importantly, between the replicated index groups in our standard protocol (A and
411 B) we recovered similar numbers of loci (797 and 645, respectively) with 549 (85.1%)
412 loci found in both datasets under our stringent filtering criteria. Moreover, when we
413 searched for the loci recovered in A in the catalog of B, and vice versa, the overlap was

414 above 99% (Table 3). This suggests that very similar sets of loci were recovered in these
415 independent assemblies, and that there was variation in the loci that passed our filtering
416 criteria. Although changing our filtering parameters led to a slight increase in the
417 proportion of overlap in the loci recovered between A and B, the total number of
418 overlapping loci increased substantially. When we accepted 20% missing data instead of
419 5%, the proportion of overlap was 86%, with 857 loci in total. When we changed the
420 minor allele frequency filter from 0.25 to 0.05 we obtained 88% overlap and a total of
421 1227 loci (Table S2, Supporting Information).
422
423 *Paternity assignments*
424 Both panels produced highly concordant results when assigning paternity, but the
425 SNP panel showed substantially higher power overall. Generally, the microsatellite loci
426 were more polymorphic, resulting in greater mean polymorphic information content (PIC)
427 for any given locus. Despite this, the SNP panel performed better because of the large
428 number of loci obtained through RAD sequencing. This greatly improved the non-
429 exclusion probabilities across different parentage assignment contexts (Table 4), and
430 reduced uncertainty in our assignments. Given the known mother, the microsatellite and
431 SNP panels assigned the same fathers to all 40 offspring with 95% confidence. When
432 paternity assignments were made without the known mother (no known candidate
433 parents), both panels again assigned fathers for all 40 offspring with 95% confidence.
434 However, 5 of these assignments were not in agreement between the two panels. For
435 these 5 cases, two candidate males had very similar LOD scores under the

436 microsatellite panel, and the assigned males did not match the males that were
437 assigned given the known mother. These (and all other) cases were resolved
438 unambiguously when the SNP panel was used, and the paternity assignments with and
439 without the known mother were in complete agreement for all offspring (Fig. 1).
440 Overall, both panels assigned 23 out of 40 nestlings (57.5%) to males that were
441 not their social father. Due to the nature of our non-random sampling of individuals for
442 this experiment, and the overall smaller sample size, this value is slightly lower than the
443 overall rate of 67.6% extra-pair young observed for all years of the study (unpublished
444 data).
445 As a measure of certainty for our assignments, we calculated the difference
446 between LOD scores for the two top-ranked males assigned to each nestling, under
447 each panel (Fig. 2). Typically, this difference was 8 – 10x higher for the SNP panel
448 (n=40, mean = 165.0) than for the microsatellite panel (n=40, mean 19.1), a reflection of
449 the much higher discriminatory power of the SNP dataset. For the SNP panel, many of
450 the second-ranked males had a strongly negative LOD score, making them extremely
451 unlikely to be the true father. This was less often true for the microsatellite panel, as the
452 second-ranked males often had positive, or just slightly negative, LOD scores. Overall
453 this result illustrates the increased discrimination power achieved by the SNP panel
454 compared to the microsatellites, which allowed us to assign paternity in cases in which
455 the microsatellite assignments remained ambiguous or (albeit rarely) misleading.
456
457 *Relatedness analysis*

The SNP panel produced simulated data that closely matched the observed allele and genotype frequencies (Pearson's correlation coefficient = 0.975). The microsatellite panel also matched well, but was not as reliable as the SNP panel (Pearson's correlation coefficient = 0.877). This resulted in better estimates of pairwise relatedness for parent-offspring using the SNP panel (Fig. 3). Overall, the SNP panel produced better simulated estimates for each degree of relatedness (Fig. 4), greatly reducing the variance around expected relatedness values (unrelated = 0, half-sib = 0.25, full-sib = 0.5, and parent-offspring = 0.5). This bolsters the confidence with which actual relationships can be discerned when calculating pairwise relatedness of a population for which there is little prior knowledge of social relationships.

Discussion

Several recent studies have rigorously investigated the use of SNPs in population genetic studies for several non-model organisms (Morin *et al.* 2004; Slate *et al.* 2010; Garvin *et al.* 2010; Heylar *et al.* 2011; Seeb *et al.* 2011), with growing support for the use of SNPs in studies of parentage (Anderson & Garza 2006; e.g. Hauser *et al.* 2011; e.g. Kaiser *et al.* 2017; e.g. Kess *et al.* 2016) and relatedness (e.g. Glaubitz *et al.* 2003; Wang 2007). SNPs have proven to perform as well, if not better than microsatellites in these types of studies. To our knowledge this is the first study to describe a universal ddRAD-seq method for use in parentage and relatedness analyses of wild populations. Our study is also the first to compare the efficiency of microsatellites versus SNPs for determining genetic relationships in a bird species that is both socially complex and

480 highly promiscuous. We show that SNPs developed from our modified ddRAD-seq
481 method are substantially more powerful than a moderate number of species-specific
482 microsatellite loci at assigning paternity and estimating relatedness among individuals.
483 Our method is highly attractive as an alternative to traditional microsatellite genotyping,
484 especially for systems where no microsatellites have been developed. This is largely
485 due to the combination of its cost and researcher time efficiency, the ease of this non-
486 species-specific method that combines the SNP discovery and screening steps, and the
487 large number of SNPs reliably recovered.

488 The total approximate materials cost for our ddRAD-seq analyses, including DNA
489 extraction, normalization of the DNA concentrations, library preparation, sequencing and
490 computational time was US $3,270.00 for 240 samples, or approximately $13.6 per
491 sample. The initial investment in oligonucleotides (i.e., primers and adaptors; see the
492 Supplementary ddRAD-seq Protocol in the Supporting Information) was US $2000 and
493 is sufficient for the analysis of thousands of samples, making the per sample cost
494 negligible. The use of a homemade MagNA in place of commercial SPRI beads provides
495 significant savings. This cost is similar to that for genotyping 240 individuals at 12
496 microsatellite loci (in 3 multiplexed PCR mixes), in a situation where the labelled primers
497 have already been designed, purchased, and tested. However, a substantial additional
498 benefit of this ddRAD-seq method is that it does not require any locus discovery or
499 development before starting. The time required for library preparation, once DNA has
500 been extracted, is modest, and once the sequence data have been obtained, SNP
501 calling for the entire dataset can be performed in less than a day through a largely

502 automated bioinformatics pipeline (see Supporting Information). Unlike manually scoring
503 peaks in traditional microsatellite genotyping analyses, the identification of SNPs is less
504 subjective and takes far fewer hours of hands-on analysis (as most is performed
505 computationally). The tools for analyzing these ddRAD data are freely available and
506 widely used (e.g., Stacks, VCFtools). Nevertheless, we note that assembling RAD loci
507 can still be challenging, and the choice of bioinformatics pipelines and specific
508 combinations of assembly parameters can influence the quality and quantity of loci
509 recovered (for detailed discussions on these issues see Eaton 2014, Mastretta-Yanes *et*
510 *al.* 2015, Shafer *et al.* 2017 and Paris *et al.* 2017).

511 For this study, our conditions and protocol allowed us to recover 411 high quality
512 SNP loci for 160 individual samples (although 240 samples were multiplexed together on
513 one lane of sequencing). However, we show that through simple variations in the size
514 selection window or the specificity of the restriction enzyme, more or fewer loci can be
515 obtained. For some applications, it could be advantageous to multiplex a greater number
516 of individuals and achieve similar coverage by aiming to recover fewer loci (e.g., using
517 EcoRI rather than MspI). Alternatively, for applications where more loci are required, the
518 size selection window could be widened and concordantly the number of individuals
519 would have to be lowered. It is also possible to vary the number of loci retained by
520 applying different bioinformatics filters. With strict filtering parameters (5% missing data,
521 minor allele frequency of 0.25 and minimum depth of coverage of 10x) we recovered
522 411 loci, which contained sufficient information to accurately assign paternity and
523 estimate relatedness among the individuals in our study. However, with only slight

524 modification of these parameters it is possible to greatly increase the number of loci
525 recovered. A total of 506 loci were retained when we allowed a minimum coverage of 5x,
526 742 with up to 20% missing data, and 910 with a minor allele frequency of 0.05 (we
527 varied one filter at a time).

528 The number of SNPs needed to perform robust parentage and relatedness
529 analyses depends on characteristics of the study population. Populations with reduced
530 genetic diversity will likely require a greater number of loci than those that are more
531 genetically diverse (Saunders *et al.* 2007; Strucken *et al.* 2016; Tortereau *et al.* 2017).
532 Obtaining more loci from the outset would aid in overcoming any issues relating to
533 population genetic diversity. Additionally, when studying species with complex social
534 systems, including for example both variable levels of genetic relatedness among
535 individuals and high rates of extra-pair fertilizations, it is imperative to obtain a sufficient
536 number of markers to discern genetic relationships robustly (Hughes 1998; Ross 2001;
537 Weinman *et al.* 2015). Our case study, using the variegated fairy-wren, shows that our
538 modified ddRAD-seq method recovers more than enough SNP loci to confidently discern
539 relationships in a species with a complex social system. Most parentage and
540 relatedness analysis programs are well equipped to handle large numbers of loci, so a
541 greater number of loci would not hinder analyses. Once an appropriate number of SNPs
542 are identified for performing robust analyses, conditions can be varied to maximize the
543 number of individuals to be genotyped. For our purposes, we conducted parentage and
544 relatedness analyses in CERVUS and the R package 'related,' respectively, to reliably
545 compare the performance of our microsatellite and SNP panels. Several other pedigree

546 reconstruction programs are readily available (e.g. COLONY, MasterBayes, and
547 Sequoia) and researchers can easily input SNP data into their preferred program (for
548 detailed comparisons of some of these programs see Karaket *et al.* 2012 and Weinman
549 *et al.* 2015). The R package, 'Sequoia' (Huisman 2017), is specifically tailored for SNP
550 data, and can reconstruct multi-generational pedigrees with as few as 100 SNPs and
551 many non-genotyped individuals. Given these considerations, 'Sequoia' may be
552 particularly useful for studies with limited social information or incomplete population
553 sampling.

554 For both paternity and relatedness analyses, our SNP panel far outperformed our
555 microsatellite panel by providing much more power and improving the overall confidence
556 for assignments. Variegated fairy-wrens are relatively easy to observe, and every nest
557 found can be assigned to a known mother by watching the female that builds the nest
558 and/or incubates the eggs. This level of knowledge may not be the norm for most study
559 systems, so we also investigated the CERVUS output for male-offspring relationships,
560 independent of known mothers. In doing so, the reliability of the SNP panel became
561 even more evident. In CERVUS, the higher the LOD score, the more likely that a given
562 male is the true father. Using SNPs, CERVUS typically output only a single male with a
563 positive LOD score, and the difference in LOD scores between the top-two ranked males
564 was dramatically different for SNP assignments (Fig. 2). When social information about
565 the known mother was excluded from the paternity analysis, the microsatellite panel
566 sometimes produced assignments that were ambiguous (two males had similar LOD
567 scores), and occasionally the wrong male was assigned paternity of the offspring. Under

568 the SNP panel, ambiguous assignments were nonexistent, and these cases were clearly
569 resolved (Fig. 1).
570 It is sometimes difficult to obtain appropriate demographic data to use in a formal
571 parentage analysis, and for many studies, this level of detail may not be necessary.
572 Population allele frequencies can be used to estimate pairwise relatedness for
573 individuals, and to reconstruct pedigrees using maximum likelihood-based methods.
574 Variance in estimates of pairwise relatedness (*r*) for known parent-offspring pairs was
575 dramatically reduced when using SNPs (Fig. 3). For our simulations, SNPs greatly
576 improved the differentiation between distributions for individuals of known degrees of
577 relatedness (Fig. 4). This is particularly important for systems with minimal demographic
578 and observational data, where these distributions can be used to determine familial
579 relationships between individuals, in conjunction with actual estimated *r*-values.
580 This protocol was designed to be universally applicable across bird species, and
581 we have successfully applied it in a range of other avian study systems (Table 5). While
582 different numbers of individuals were used in each study, and therefore different
583 numbers of loci were recovered, in all cases paternity was confidently assigned to
584 nestlings using CERVUS (unpublished results). We note that when the number of
585 individuals genotyped was larger (we have so far tested up to 480 individuals), the
586 number of loci recovered after filtering was smaller (as low as 135 SNPs with stringent
587 filtering parameters). Our controls designed to assess repeatability (e.g., compare group
588 A and B or D and E in Tables 2 and 3) suggest that there is a high degree of overlap
589 among replicates, but also variation in which loci pass our filters. In cases in which a

590 larger number of loci are needed to accurately assess paternity or estimate relatedness,
591 some of the filtering parameters may be relaxed. For example, for a set of 373
592 Variegated fairy-wren individuals (Table 5) the number of loci retained increased from
593 157 to 410 when 20% missing data was allowed instead of 5%. Accordingly, our protocol
594 is suitable for long-term studies in which samples are accumulated across several years,
595 especially if not all individuals need to be compared simultaneously (e.g. non-
596 overlapping generations or individuals from different years). It is also likely that this
597 protocol can be applied successfully for studies (short or long-term) where thousands of
598 individuals need to be compared at one time. This remains to be shown, though, and in
599 such cases it may be more appropriate to use techniques based on microarrays (see
600 Fernández *et al.* 2013, Liu *et al.* 2016, and Tortereau *et al.* 2017).

601 Applying this general protocol to many non-avian taxa will simply require ensuring
602 that specific restriction enzymes and fragment size windows are chosen appropriately.
603 The size of the genome and the number of individuals multiplexed will have to be taken
604 into consideration to achieve the desired coverage.

605 In summary, our ddRAD-seq method provides a cost effective and robust way to
606 identify SNPs for use in studies utilizing parentage and relatedness analyses. Our
607 experiment shows that a majority of the same SNPs can be obtained across groups,
608 using the same size selection windows and restriction enzymes. Future individuals can
609 be genotyped and incorporated to the analysis by re-running the Stacks pipeline. Using
610 a bird exhibiting great social complexity, and high promiscuity, we have shown that

SNPs identified by ddRAD-seq are more effective at assigning paternity and estimating relatedness than highly polymorphic, species-specific microsatellite loci.

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Data Accessibility

The raw data used in this manuscript will be stored in the Dryad Digital Repository upon acceptance.

Author Contributions

D.J.T designed the study, collected field data, performed microsatellite development and analysis, conducted parentage and relatedness analyses, and drafted the manuscript with help from all co-authors. B.G.B and L.C. designed the study, and performed SNP discovery and analysis. M.S.W and I.J.L. helped design the study, and secured funding.

Tables

Table 1: Experimental design. Index groups 9-12 were included in our sequencing run to assess how changes in our molecular protocol impacted the number of loci recovered. Therefore, we selected the same 20 individuals for these four index groups to reduce the possible sources of variation.

Number of samples (index groups)	Enzymes	Size selection interval	Group
160 samples (8 index groups, index 1-8)	SbfI - MspI	450 - 600 bp	All
20 samples (Index 1)	SbfI - MspI	450 - 600 bp (replicate of above)	A
20 samples (Index 9)	SbfI - MspI	450 - 600 bp (replicate of above)	B
20 samples (index 10)	SbfI - MspI	400 - 700 bp (wide size selection)	C
20 samples (index 11)	SbfI - EcoRI	450 - 600 bp (infrequent 3' cutter)	D
20 samples (index 12)	SbfI - EcoRI	450 - 600 bp (replicate of above)	E

Table 2. Overlap in the RAD loci that were obtained while varying different steps of the protocol (size selection and restriction enzymes). The diagonal indicates the total number of loci recovered for each treatment. Values above the diagonal represent the percent overlapping loci between groups (relative to the group with the smallest number of loci), while values below the diagonal list the number of loci that were overlapping between groups.

	All	**A**	**B**	**C**	**D**	**E**
All	411	85.4	78.1	79.1	1.2	1.0
A	351	797	85.1	75.8	0.8	0.8
B	321	549	645	83.4	2.0	2.3
C	325	604	538	1440	4.2	3.9
D	5	15	12	25	596	68.4
E	4	13	12	20	353	516

All: 160 samples; Sbf1/Msp1; 450-600 bp.
A: 20 samples; Sbf1/Msp1; 450-600 bp (Index 1).
B: 20 samples; Sbf1/Msp1; 450-600 bp (Index 9).
C: 20 samples; Sbf1/Msp1; 400-700 bp (Index 10).
D: 20 samples; SbfI/EcoRI; 450-600 bp (Index 11).
E: 20 samples; SbfI/EcoRI; 450-600 bp (Index 12).

909 Table 3. Overlap in filtered RAD loci and those present in the catalogs from the
910 different assemblies. The filtered loci from the groups in the different rows were aligned
911 against databases generated from the catalogs of the groups in the columns. The
912 numbers in the table are the percent of filtered loci from each group that match loci that
913 are in the catalog of the target database. The diagonal contains the total number of loci
914 recovered for each group and the total number of loci in each catalog are 49662 (All),
915 27085 (A), 25521 (B), 29554 (C), 11823 (D), 16724 (E).
916

	All	**A**	**B**	**C**	**D**	**E**
All	411	99.5	99.3	99.0	5.6	9.2
A	99.2	797	99.1	99.1	7.9	11.9
B	99.2	99.1	645	99.1	7.9	12.6
C	98.5	88.7	89.8	1440	7.4	11.4
D	37.1	22.8	21.8	22.5	596	99.7
E	42.8	21.3	19.0	20.2	96.3	516

917
918
919 Table 4. Marker characteristics. He: Expected heterozygosity; Ho: Observed
920 heterozygosity; PIC: Polymorphic information content.
921

Marker Panel	Number of loci	Mean proportion loci typed	Mean alleles per locus	Mean H_e	Mean H_o	Mean PIC	Nonexclusion probability (first parent)	Nonexclusion probability (second parent)	Nonexclusion probability (parent pair)
Microsatellites	12	0.99	14.17	0.77	0.76	0.74	1.9×10^{-4}	1.9×10^{-6}	1.5×10^{-10}
SNPs	411	0.98	2.00*	0.45	0.45	0.35	5.2×10^{-20}	6.9×10^{-35}	1.0×10^{-55}

922 *Only biallelic SNPs were retained. If a locus had 3 alleles across the population, it was filtered from the dataset.
923
924
925 Table 5: Summary information for ddRAD-seq studies performed to investigate
926 parentage in other bird species. Only SNPs with up to 5% missing data, with a minimum
927 coverage of 10x and a minor allele frequency of 0.25 were retained. HWE: Hardy-
928 Weinberg equilibrium.
929

Species	**Scientific name**	**Number of individuals**	**Number of loci**	**Number of loci in HWE**
Variegated fairy-wren*	*Malurus lamberti*	373	234	157
Hispaniolan woodpecker**	*Melanerpes striatus*	288	179	135
Northern Red-billed hornbill	*Tockus erythrorhynchus*	40	475	414
Von der Decken's hornbill	*Tockus deckeni*	112	490	410
Sapayoa	*Sapayoa aenigma*	6	672	671
Red-backed fairy-wren***	*Malurus melanocephalus*	480	329	167

930

* Two independent ddRAD-seq experiments were performed – one with 160 samples (those from the current study) and a second with 213 samples. After filtering and demultiplexing, the data from 373 samples were combined for denovo assembly and SNP identification.

** Samples were run in two experiments and combined, one with 240 samples and the other with 48.

*** Two independent ddRAD-seq experiments were run on each set of 240 samples.

Figures

Figure 1. Resolved paternity assignments for 5 nestlings with ambiguous assignments under the microsatellite panel, but not with the SNP panel. Each panel in the graph represents an individual offspring, and the two top-ranked males are depicted as a triangle and a circle, respectively. Lines connecting like shapes show the change in LOD score for each male, using each marker type (microsatellites versus SNPs). Note that the y-axis scale varies among panels in the graph.

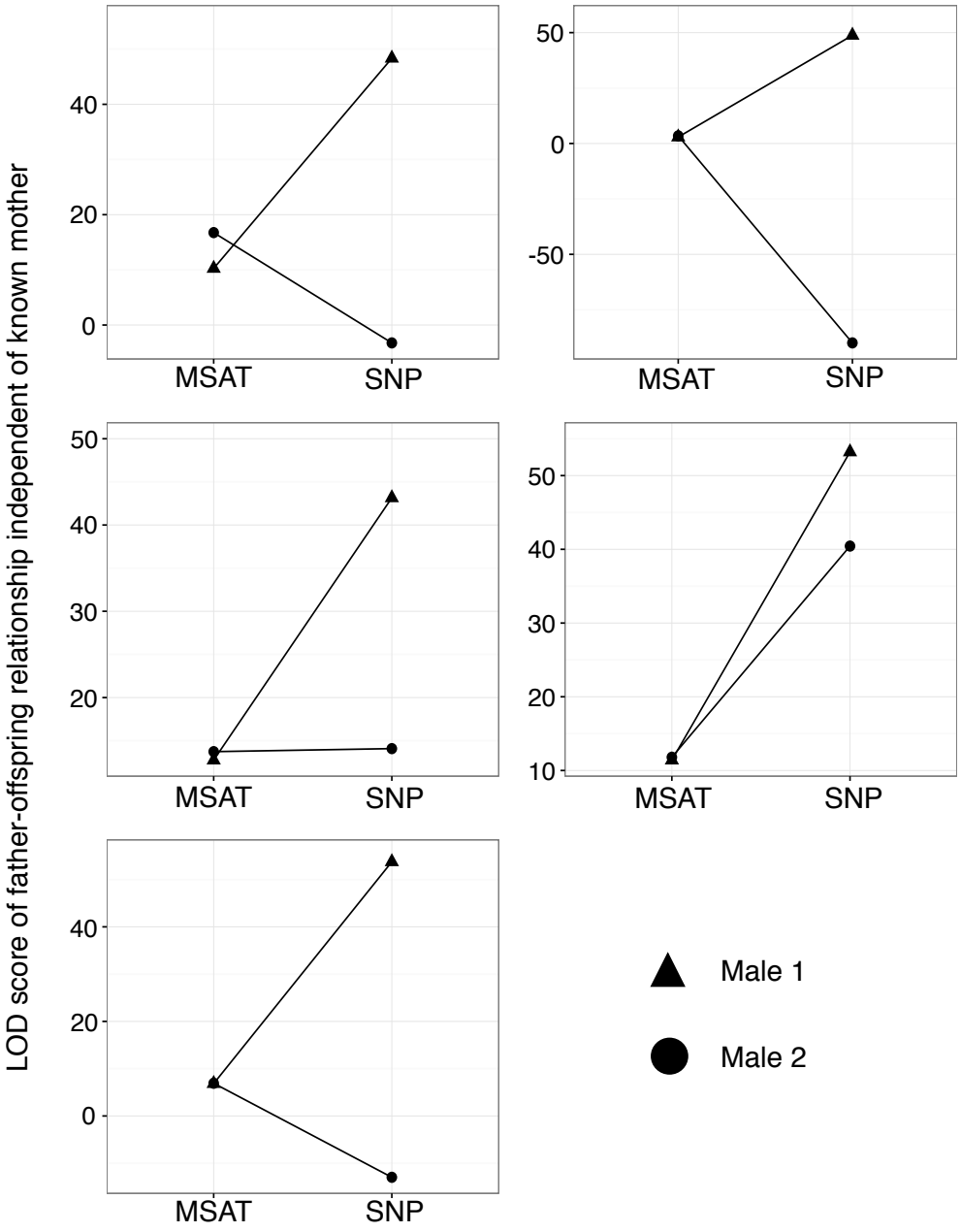
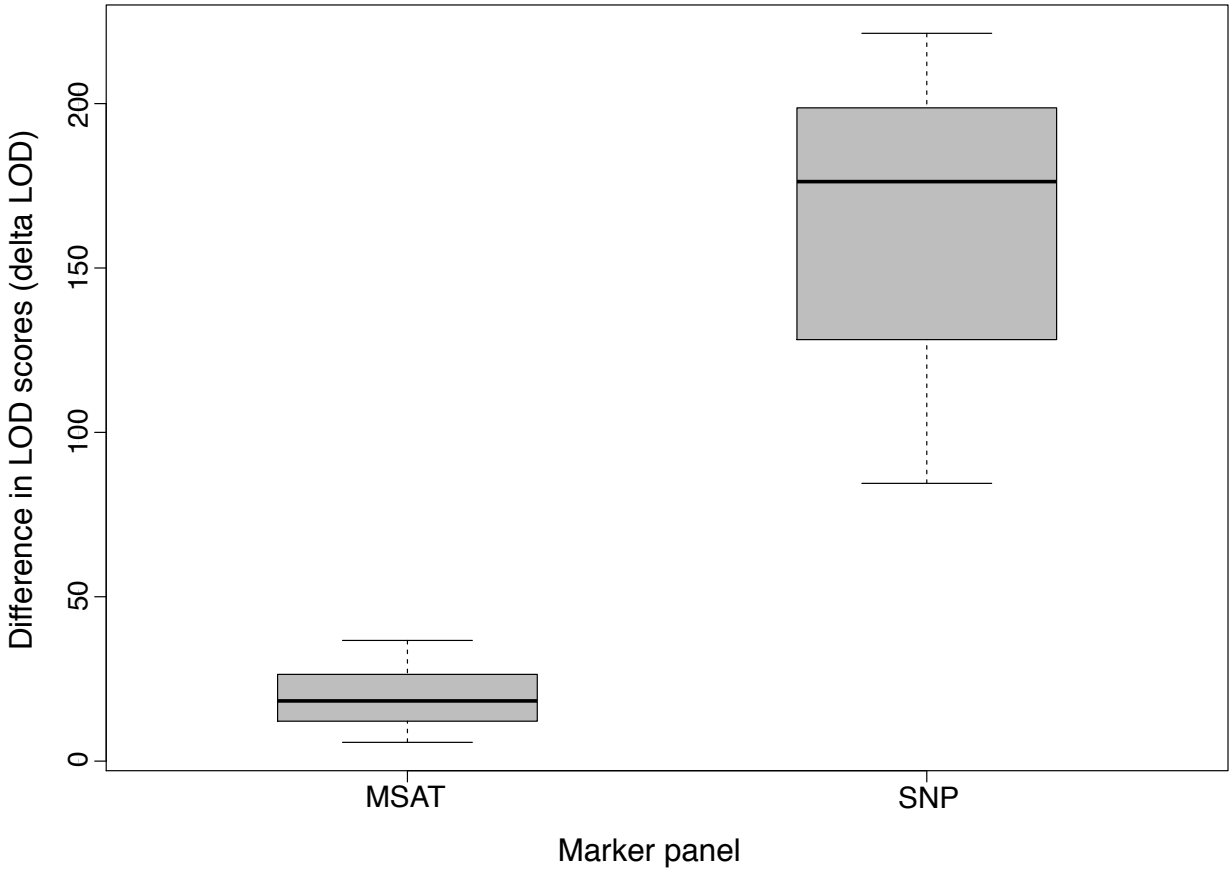
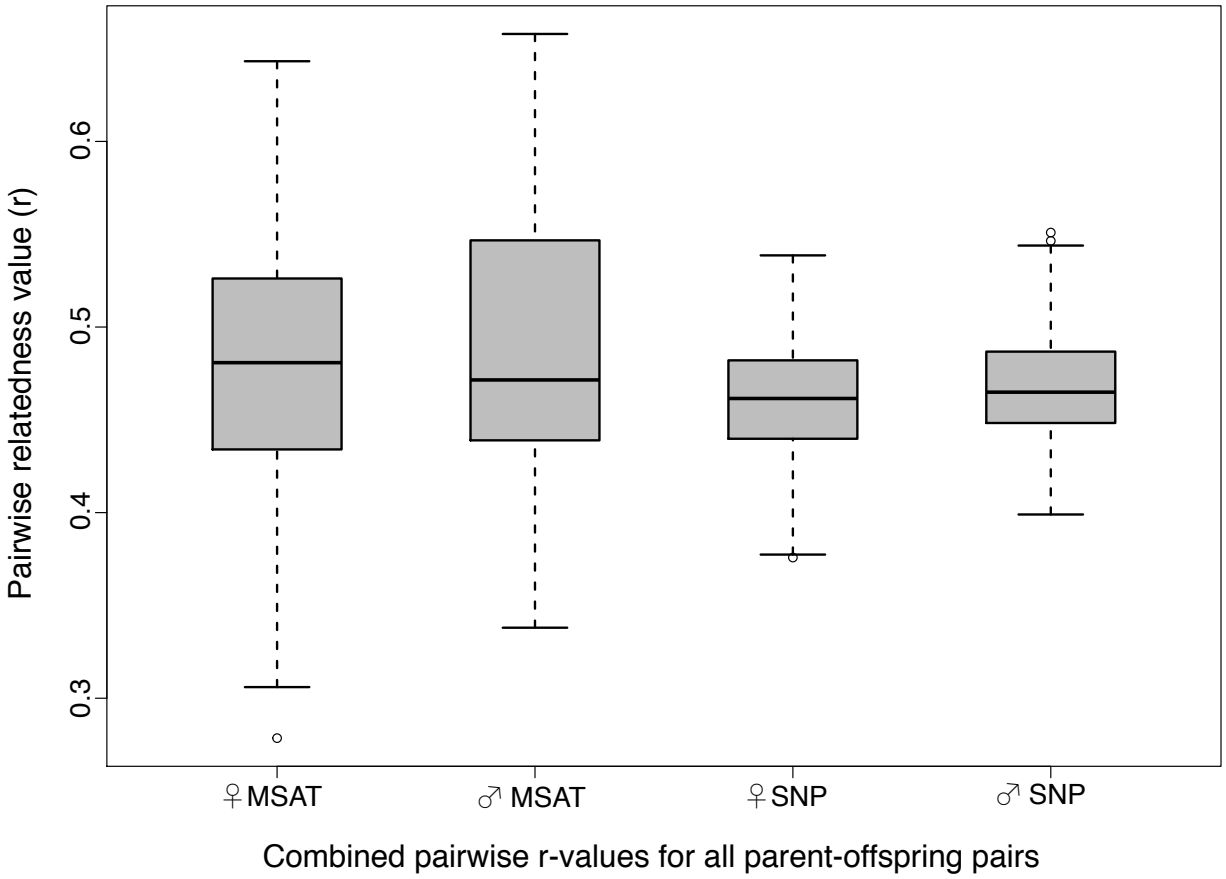


Figure 2. Difference in CERVUS LOD scores (delta LOD) between the most likely father of a nestling and the second possible father in the population, for both marker panels.



1001 Figure 3. Box plot of pairwise relatedness values for all parent-offspring (40 mother-
1002 offspring and 40 father-offspring) relationships, using population allele frequencies from
1003 each marker panel.
1004



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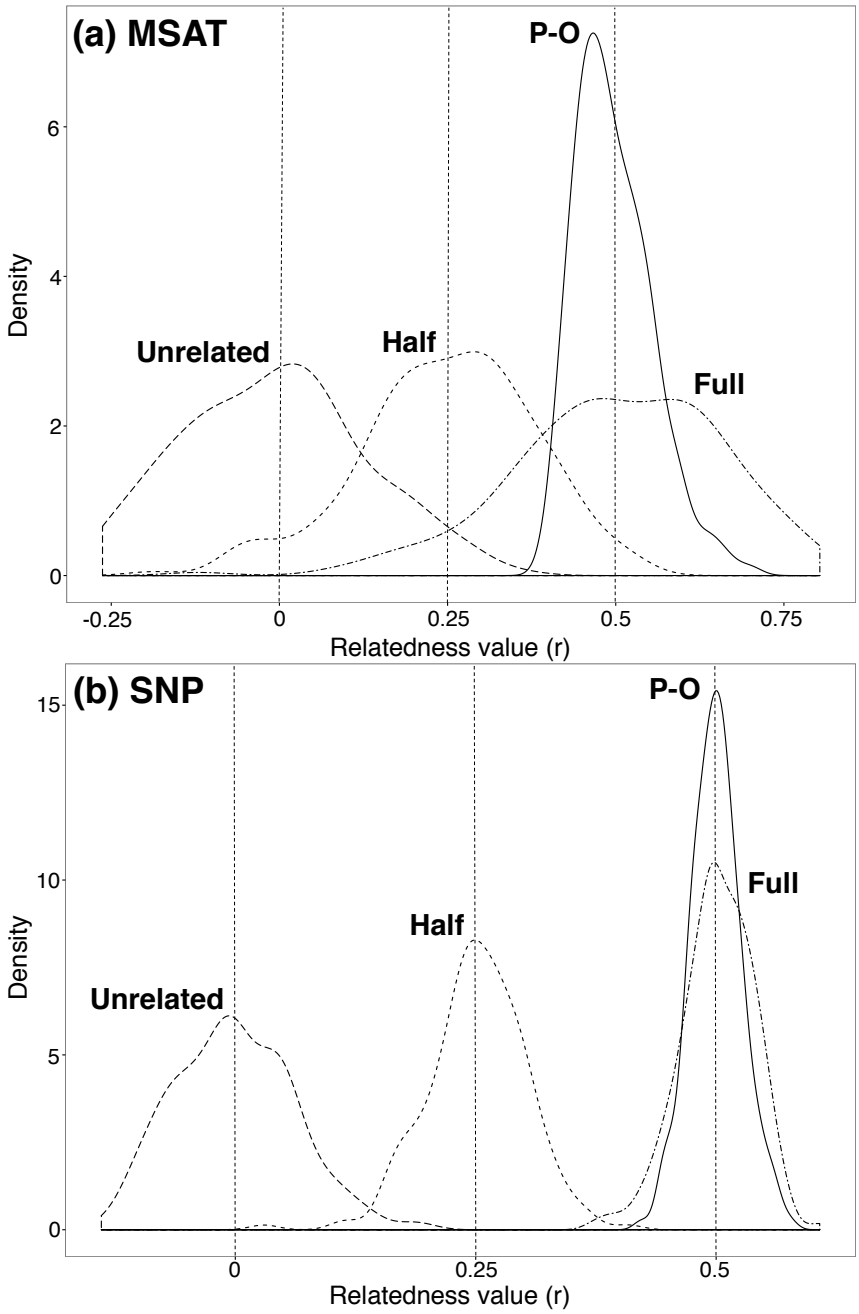
Figure 4. Density plots of relatedness values for simulated pairs of known relatedness (unrelated, half-sibling, full-sibling, and parent-offspring) using population allele frequencies from each marker panel (a. MSAT; b. SNP). Overlap in distributions indicates the overlap between relatedness value estimators for pairs of individuals of different relationships. The spread of each distribution indicates the reliability of observed relatedness values based on their deviation from expected relatedness values (Unrelated = 0, Half-sib = 0.25, Full-sib = 0.5, and Parent-offspring (P-O) = 0.5, denoted by vertical dashed lines).