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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Running title: SNPs outperform microsatellite loci

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 substantially 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, especially in more distantly related taxa (Galbusera 2000; Decroocq *et al.* 2003; Hedgecock *et al.* 2004; Primmer *et al.* 2005). Traditional PCR-based microsatellite assays incur substantial financial and lab-bench time investments. The manual scoring of microsatellite alleles also requires substantial researcher time, and can involve various forms of error arising from alleles that have more than one clearly defined peak, allelic drop-out and null allele issues, and the various sources of human error that are inherent in any complicated workflow (Pemberton *et al.* 1995; Hedgecock *et al.* 2004; Hoffman & Amos 2005; Kalinowski *et al.* 2007).

Many of these limitations are less severe in assays based on single-nucleotide polymorphisms (SNPs), which require fewer steps and have greater automation (Gut 2001; Syvänen 2001; Seeb *et al.* 2011; Davey *et al.* 2011). SNPs are appropriate alternatives for studies of parentage and relatedness data because they are abundant in the genome, have low mutation rates (Brumfield *et al.* 2003; Morin *et al.* 2004), and can be scored semi-automatically (Garvin *et al.* 2010; Guichoux *et al.* 2011). In comparison to microsatellite-based relationship tests, the primary limitation of SNPs is that SNPs are typically biallelic, whereas microsatellite loci are often multiallelic, and hence the statistical power of SNP loci for discriminating parentage and relatedness is far lower on a per-locus basis (Ball *et al.* 2010). Compared to highly variable microsatellite loci, a substantially higher number of SNP markers is therefore required to achieve appropriate power in parentage and relatedness studies (Glaubitz *et al.* 2003; Morin *et al.* 2004; Coates *et al.* 2009).

Recently, the application of SNPs for use in analyses of parentage, relatedness, and overall population structure has received greater attention (Glaubitz *et al.* 2003; Anderson & Garza 2006; Coates *et al.* 2009). Studies in birds (Cramer *et al.* 2011; Weinman *et al.* 2014; Kaiser *et al.* 2016), fish (Hauser *et al.* 2011), and several domesticated taxa (Tokarska *et al.* 2009; Fernández *et al.* 2013) have developed SNP panels with a sufficient number of SNPs to attain a comparable, if not better, level of resolving power as highly polymorphic microsatellite panels. While each of these studies manage to identify powerful SNP panels, the SNP genotyping methods used are often labor intensive, requiring a significant amount of preparatory work at the discovery stage

prior to genotyping of large numbers of individuals. Many of these methods also rely on reference genomes (Anderson & Garza 2006; Heylar *et al.* 2011), or other genomic resources (Fernández *et al.* 2013; Weinman *et al.* 2014; Kaiser *et al.* 2016) (e.g. transcriptome, SNP microarray), for SNP identification. Ultimately, this has afforded several beneficial examples of the utility of SNPs for parentage and relatedness analyses, but without an efficient, universal method of SNP discovery and identification.

Restriction site-associated DNA sequencing (RAD-seq) is widely used in molecular genetic studies (Davey & Blaxter 2010; Etter *et al.* 2012; Puritz *et al.* 2014), particularly for linkage and quantitative trait locus (QTL) mapping (Baird *et al.* 2008), genome wide association studies (Davey *et al.* 2011), and phylogeography (Andrews *et al.* 2016). RAD-seq uses a restriction enzyme to fragment and sample a fraction of a genome; as it identifies SNPs with no prior knowledge of the genome, it provides a more universal method of SNP discovery (Willing *et al.* 2011). Double-digest restriction site-associated DNA sequencing (ddRAD-seq) allows for selection of an even smaller fraction of the genome through the combined use of two restriction enzymes, affording the ability to target a smaller total number of SNPs in a greater number of individuals (Peterson *et al.* 2012; Puritz *et al.* 2014; Kess *et al.* 2016). This ability, in concert with the fact that no prior knowledge of the genome is needed, makes ddRAD-seq an attractive method of simultaneous SNP discovery and screening for use in discerning genetic relationships among individuals.

Here, we describe a ddRAD-based approach to the simultaneous discovery and screening of high numbers of SNP loci with high power for testing questions about

parentage and relatedness. These protocols are optimized to generate an appropriately robust set of SNP markers for 240 individuals per run, to be repeatable across runs to allow the combination of SNP datasets generated at different times, and to be universally applicable to birds (and with small modifications, to other organisms) without requiring a species-specific marker discovery step. We validate these methods by conducting a SNP-based parentage and relatedness study in the highly promiscuous, and socially complex, variegated fairy-wren (*Malurus lamberti*). We compare the results with previously generated paternity assignments and relatedness information, based on microsatellite screens of the same fairy-wren individuals and social groups. To illustrate the broad utility of this method we report summary statistics for equivalent studies of parentage in a variety of other species that collectively span much of the phylogenetic diversity of living birds.

Methods & Materials

Study Population

The variegated fairy-wren, endemic to Australia, is a cooperatively breeding bird that lives in social groups composed of kin and non-kin (Schodde 1982; Rowley & Russell 1997). Male dispersal is limited, and rates of extra-pair fertilizations (EPFs) are high (~68% of all young, assessed with a panel of 12 species-specific microsatellites; DJ Thrasher, unpublished data). We intensively monitored a color-banded population of the nominate subspecies, *M. l. lamberti*, on Lake Samsonvale (27^{O}16' S, 152^{O} 41' E), 30 km northwest of Brisbane, Queensland, Australia, from 2012 – 2016. The population

153 ranges from about 250-300 adults depending on year-to-year conditions. The study site
154 is bounded on most sides by Lake Samsonvale, and on its westernmost side by a major
155 highway, which increases our confidence in sampling most, if not all, of the adults in the
156 population. We also monitored all nesting attempts to measure, mark, and collect blood
157 samples from nestlings 6 days after hatching. Blood samples were immediately stored in
158 lysis buffer (White & Densmore 1992), and genomic DNA was later extracted using
159 Qiagen DNeasy Blood and Tissue kits. DNA concentration was determined using the
160 Qubit dsDNA BR Assay Kit and the Qubit® Fluorometer (Life Technologies) following
161 the manufacturers protocol.
162
163 *Microsatellite development and genotyping*
164 We previously developed twelve polymorphic microsatellite loci for the variegated
165 fairy-wren (Table S1, Supporting information) following methods described previously in
166 Nali *et al.* (2014). Briefly, we extracted genomic DNA from blood in lysis buffer from eight
167 adults in our study population, enriched the mix of DNA with repetitive sequences to
168 develop an enriched microsatellite library, and conducted an Illumina MiSeq sequencing
169 run. From this pool of sequences, we optimized twelve loci that amplified well using
170 polymerase chain reaction (PCR), were polymorphic, and exhibited clearly defined
171 peaks for genotyping. We designed three multiplexed PCRs for genotyping, and each
172 amplification reaction contained 1ul of genomic DNA of varying concentrations (1 ng-40
173 ng per 1 ul). PCR products were combined with the GeneScan 500 base pair LIZ
174 internal size standard for size-sorting using a 3730 DNA Analyzer. We used Geneious

175 version 8.0 (Kearse *et al.* 2012) to score alleles. The program automatically identifies
176 alleles at each locus, and we manually inspected allele calls to minimize genotyping
177 error. In total, we genotyped 287 adults and 482 nestlings from 226 nests sampled
178 during the 2012-2016 breeding seasons.
179
180 *ddRAD sequencing*
181 We selected a subset of the individuals genotyped for microsatellite loci (120
182 adults and 40 nestlings) for use in our ddRAD-seq experiment and subsequent
183 analyses. To assess the reliability of our SNP panel for parentage and relatedness
184 analysis, we chose representative nestlings from all the years of our study. Typically, we
185 selected one nestling from any individual nest. In a few cases, we selected two nestlings
186 that prior microsatellite analysis had assigned to the same mother but different fathers.
187 For each nestling, we included the mother, the social father, and the genetic father as
188 assigned by previous microsatellite analysis. Our pool of candidate parents included 24
189 mothers and 78 putative fathers, and 18 randomly selected individuals of both sexes, for
190 a total of 120 adults.
191 Our ddRAD-seq protocol is adapted from Peterson et al. (2012) (see Supporting
192 information for a detailed protocol). Briefly, for each individual, 100ng - 500ng of DNA
193 (20ul of DNA between concentrations of 5ng/ul - 25ng/ul) were digested with either SbfI
194 and MspI, or SbfI and EcoRI (NEB), and ligated with one of 20 P1 adapters (each
195 containing a unique inline barcode) and a P2 adapter (P2-MspI or P2-EcoRI). After
196 digestion and ligation these samples were pooled in groups of 20 (each with a unqiue P1

adpater) and purified using 1.5X volumes of homemade MagNA made with Sera-Mag Magnetic Speed-beads (FisherSci) as described by Rohland & Reich (2012). Fragments of between 450 bp and 600 bp were selected using BluePippin (Sage Science) by the Cornell University Biotechnology Resource Center (BRC). Following size selection, index groups and Illumina sequencing adapters were added by performing 11 PCR cycles with Phusion DNA Polymerase (NEB). These reactions were cleaned up with 0.7x volumes of MagNA and pooled in equimolar ratios to create a single library for sequencing on one lane of Illumina HiSeq 2500 (100bp single end, performed by BRC).

By replicating 20 samples (run as a separate index group) with a wider BluePippin size selection range of 400-700 bp, we explored the inherent trade-off between the number of samples sequenced on a lane at a given coverage threshold and the number of SNPs recovered per sample. We similarly explored the effects of using a less frequent restriction enzyme digest (by substituting the 6 bp cutter EcoRI in place of the 4 bp cutter MspI) to generate a smaller total number of fragments in our size range, which in turn should increase the sequencing coverage of the loci screened.

To assess repeatability across index groups, we replicated two index groups, each comprised of 20 samples: one index group from the standard protocol, and the index group generated with the rare-cutter EcoRI enzyme (Table 1). We multiplexed the resulting 240 samples in 12 index groups (with 20 individuals each) which were pooled into a final library that was sequenced on one lane of an Illumina HiSeq 2500, producing 220,300,739 100 bp single end reads (see Table 1).

SNP data analysis

Quality filtering and demultiplexing

After the quality of the reads was assessed using FASTQC version 0.11.5 (www.bioinformatics.babraham.ac.uk/projects/fastqc), we trimmed all sequences to 97bp using fastX_trimmer (FASTX-Toolkit) to exclude low quality calls near the 3' of the reads. We subsequently removed reads containing a single base with a Phred quality score of less than 10 (using fastq_quality_filter). We additionally removed sequences if more than 5% of the bases had a Phred quality score of less than 20. Using process_radtags module from the Stacks version 1.37 pipeline (Catchen *et al.* 2013), we demultiplexed the reads to obtain files with sequences that were specific to each individual.

De novo assembly of RAD loci

Because we do not have a sequenced genome for the variegated fairy wren or a close relative – which is likely to be the case for many non-model organisms involved in parentage studies – we assembled the sequences de novo using the Stacks pipeline (Catchen *et al.* 2013). First, we used denovo_map.pl to assemble the reads into a catalog allowing a minimum stack depth of 5 (m parameter), up to 5 mismatches per locus within an individual (M paramenter), and 5 mismatches between loci of different individuals when building the catalog (n parameter). We ran the rxstacks module to filter loci with a log likelihood of less than -50 (lnl_lim -50) or that were confounded in at least

25% of the population (conf_lim 0.25). We then built a new catalog by rerunning cstacks and obtained individual genotype calls with sstacks.

SNP filtering

SNPs were exported using the populations module of the Stacks pipeline. All of our samples were grouped in one population and a locus was exported if it was present in 95% of the individuals in this population (r parameter) at a stack depth of at least 10 (m parameter). The data were restricted to the first SNP per locus (--write_single_snp), and a minor allele frequency of at least 0.25 was required to process a nucleotide site (--min_maf).

Using vcftools version 0.1.14 (Danecek *et al.* 2011), we removed loci that were not in Hardy-Weinberg equilibrium (---hwe). We obtained a variant call format file that was converted to structure format in PGD Spider version 2.0.5.0 (Lischer & Excoffier 2012) and further modified to a format compatible with CERVUS version 3.0.7 (Kalinowski *et al.* 2007) using a custom perl script.

Parentage Analysis

We used CERVUS version 3.0.7 (Kalinowski *et al.* 2007) to assign paternity for all nestlings using our microsatellite and SNP datasets separately. CERVUS uses a two-step, likelihood-based approach to assign parentage. First, CERVUS compares each offspring's genotype to that of a candidate parent and a random individual in the population to calculate a likelihood ratio. This relationship is presented as an LOD score,

which is simply the natural logarithm of the calculated likelihood ratio. Positive LOD scores indicate that a candidate parent is much more likely to be the true parent, whereas negative LOD scores indicate that the candidate parent is highly unlikely to be a true parent. Second, CERVUS conducts a simulation of parentage analysis based on population allele frequencies and the proportion of potential parents included in the analysis. The simulation accounts for the possibility of unsampled parents, missing data, and genotyping errors. Considering these parameters, the simulation calculates critical LOD scores by comparing the LOD distributions of the most likely parent and all other candidate parents. The critical LOD score is used to determine the confidence (95% or 80%) of each parentage assignment.

CERVUS allows for different types of parentage analysis, including parent-pair (sexes known or unknown), maternity (known father, but not mother), and paternity (known mother, but not father). Variegated fairy-wrens at Lake Samsonvale are relatively easy to observe, and we were able to assign known mothers behaviorally. We subsequently confirmed this with microsatellite analyses: females that built and attended a nest throughout incubation were always the mothers of the nestlings in that nest. In many systems, a comparable level of demographic knowledge may not be available, so a marker set must be powerful enough to assign parentage with minimal social information. To investigate the broader utility of our ddRAD-seq method, we conducted analyses that relied on the inclusion of the known mother, in addition to analyses independent of the known mother, which were based only on the father-offspring relationship. We simulated paternity assignments for 10,000 offspring to determine

284 critical LOD scores, using slightly different input parameters for each panel
285 (microsatellites and ddRAD sequencing derived SNPs). Simulations for both used the
286 following parameters: 78 candidate males, 95% of candidate males sampled, estimated
287 error rate of 0.01 for mistyped loci and likelihood scores. The proportion of loci typed
288 across all individuals was different for both panels: 0.997 for the microsatellite
289 simulation, and 0.961 for the SNP simulation.

290 For both paternity analyses, we used the trio LOD score and the father-offspring
291 LOD score from CERVUS to make assignments. The trio LOD score was calculated by
292 comparing the genotypes of the candidate male and offspring, relative to that of the
293 known mother. The father-offspring LOD score only accounts for the relationship of the
294 candidate male and the offspring, independent of the known mother. CERVUS ranked
295 candidate males by LOD scores in each category, and the highest-ranking males were
296 assigned as fathers. These rankings should be in agreement, but ambiguous
297 assignments (different top-ranking males assigned in each category) may occur when
298 multiple candidate male genotypes closely match an offspring's genotype.

299 We assessed each CERVUS assignment to determine whether it was plausible,
300 and whether the assigned male was the social father or an extra-pair sire. Our criteria for
301 accepting assignments differed slightly for microsatellites and SNPs. For microsatellites,
302 we automatically accepted the CERVUS assignment if the highest-ranking male was in
303 agreement for both the trio LOD and the father-offspring LOD, and if the number of
304 mismatches between the assigned male and the offspring was ≤ 1 (8% of 12 loci). For
305 SNPs, we also accepted the assignment if the highest-ranking males by LOD score type

were in agreement, and an allowable number of mismatches were not exceeded. However, for SNPs, our allowable number of mismatches was based on the observed maximum number of mismatches between a known mother and her known offspring (max. = 7, mean = 3.4, 2% of 411 loci). For both panels, we accepted the social father as the genetic sire if he met these respective criteria. If the social father mismatched the offspring at higher numbers, or had negative LOD scores, the offspring was considered sired by an extra-pair father. We accepted assignments of extra-pair fathers using the same criteria outlined above. We did not observe cases in which an offspring could not be assigned to either its social father, or an extra-pair sire.

Relatedness Analysis

We used the package, "related" (Pew *et al.* 2015), in R version 3.2.5 (R Core Team 2016) to estimate pairwise relatedness (r) between all pairs of individuals in this study. This package accounts for genotyping errors, missing data, and can estimate relatedness using any of seven different estimators (4 non-likelihood-based, and 3 likelihood-based). "related" includes the function, **compareestimators**, which tests the performance of different estimators on simulated data that share the same characteristics as the real data. The program uses an allele frequency file to generate simulated pairs of individuals of known relatedness, and automatically estimates relatedness using four of the most commonly used estimators (all non-likelihood-based). The function calculates a correlation coefficient between observed and expected values, to evaluate which estimator performs best with the data set. Using **compareestimators**

328 to generate 200 simulated pairs of individuals for each degree of relatedness (i.e., half-
329 sib, full-sib, parent-offspring, unrelated), we determined that the Wang (2002) estimator
330 performed best for both our microsatellite and SNP datasets. We obtained point
331 estimates of relatedness using the Wang (2002) estimator, and evaluated all parent-
332 offspring relationships that were previously determined in our parentage analysis.
333 "Related" also evaluates how well different marker sets resolve degrees of
334 relatedness, given simulated genotypes based on allele frequency files. For both panels,
335 we used the **familysim** function to generate 200 pairs of individuals for each degree of
336 relatedness. We then used the **coancestry** function to analyze all pairwise relatedness
337 values with the Wang (2002) estimator. We created density plots representing
338 histograms of the relatedness values. These plots show the overlap in relatedness
339 values for degree of relatedness, and we used them to infer how well each panel
340 performed at discerning different relationships.
341
342 **Results**
343 *SNP development and analysis*
344 After trimming, filtering and demultiplexing the data, we retained a total of
345 109,524,874 reads across all index groups, with an average of approximately 9,000,000
346 reads per index group. Two individuals failed (i.e., had less than 66,000 reads each; one
347 individual from each of two index groups) and were excluded from further analysis. The
348 number of reads per sample for the remaining individuals ranged from 213,544 to
349 810,966 (mean = 459,726 ± 118,771 std. dev.).

Further analysis using the population program from stacks identified loci with at least 10X coverage, present in 95% of the individuals, and a minimum allele frequency greater than 25%. We further retained only those loci that were in Hardy-Weinberg equilibrium. When performing analyses on all 160 individuals from the primary comparison runs (Table 1), we identified 411 loci that fulfilled these criteria and were used for downstream analyses.

We varied two aspects of the ddRAD-seq protocol to assess the number of loci recovered and the reproducibility of the method. As expected, both a reduction in the range of fragment sizes selected during the construction of the library (compare index 1 (150 bp size selection) and index 10 (300 bp size selection)) or the use of the less frequent cutter, EcoRI (index 11 and 12), resulted in fewer loci recovered (Table 3). Those from the EcoRI digest were non-overlapping with those from the MspI index groups. More importantly, between the replicated index groups in our standard protocol (index 1 and index 9) we recovered similar numbers of loci (797 and 645, respectively) with 549 (85.1%) loci found in both datasets (under our stringent filtering criteria).

Paternity assignments

Both panels produced highly concordant results when assigning paternity, but the SNP panel showed substantially higher power overall. Generally, the microsatellite loci were more polymorphic, resulting in greater mean polymorphic information content (PIC) for any given locus. Despite this, the SNP panel performed better because of the large number of loci obtained through RAD sequencing. This greatly improved the non-

exclusion probabilities across different parentage assignment contexts (Table 2), and reduced uncertainty in our assignments. Given the known mother, the microsatellite and SNP panels assigned the same fathers to all 40 offspring with 95% confidence. When paternity assignments were made without the known mother (no known candidate parents), both panels again assigned fathers for all 40 offspring with 95% confidence. However, 5 of these assignments were not in agreement between the two panels. For these 5 cases, two candidate males had very similar LOD scores under the microsatellite panel, and the assigned males did not match the males that were assigned given the known mother. These (and all other) cases were resolved unambiguously when the SNP panel was used, and the paternity assignments with and without the known mother were in complete agreement for all offspring (Fig. 1).

Overall, both panels assigned 23 out of 40 nestlings (57.5%) to males that were not their social father. Due to the nature of our non-random sampling of individuals for this experiment, and the overall smaller sample size, this value is slightly lower than the overall rate of 67.6% extra-pair young observed for all years of the study (unpublished data).

As a measure of certainty for our assignments, we calculated the difference between LOD scores for the two top-ranked males assigned to each nestling, under each panel (Fig. 2). Typically, this difference was 8 – 10x higher for the SNP panel (n=40, mean = 165.0) than for the microsatellite panel (n=40, mean 19.1), a reflection of the much higher discriminatory power of the SNP dataset. For the SNP panel, many of the second-ranked males had a strongly negative LOD score, making them extremely

394 unlikely to be the true father. This was less often true for the microsatellite panel, as the
395 second-ranked males often had positive, or just slightly negative, LOD scores. Overall
396 this result illustrates the increased discrimination power achieved by the SNP panel
397 compared to the microsatellites, which allowed us to assign paternity in cases in which
398 the microsatellite assignments remained ambiguous or (albeit rarely) misleading.
399
400 *Relatedness analysis*
401 The SNP panel produced simulated data that closely matched the observed allele
402 and genotype frequencies (Pearson's correlation coefficient = 0.975). The microsatellite
403 panel also matched well, but was not as reliable as the SNP panel (Pearson's
404 correlation coefficient = 0.877). This resulted in better estimates of pairwise relatedness
405 for parent-offspring using the SNP panel (Fig. 3). Overall, the SNP panel produced
406 better simulated estimates for each degree of relatedness (Fig. 4), greatly reducing the
407 variance around expected relatedness values (unrelated = 0, half-sib = 0.25, full-sib =
408 0.5, and parent-offspring = 0.5). This bolsters the confidence with which actual
409 relationships can be discerned when calculating pairwise relatedness of a population for
410 which there is little prior knowledge of social relationships.
411
412 **Discussion**
413 Several recent studies have rigorously investigated the use of SNPs in population
414 genetic studies for several non-model organisms (Morin *et al.* 2004; Slate *et al.* 2010;
415 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.* 2016; 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 of birds. Our study is also the first to compare the efficiency of microsatellites versus SNPs for determining genetic relationships in a species that is both socially complex, and highly promiscuous. We show that SNPs developed from our modified ddRAD-seq method are substantially more powerful than a moderate number of species-specific microsatellite loci at assigning paternity and estimating relatedness among individuals. Our method is highly attractive as an alternative to traditional microsatellite genotyping, especially for systems where no microsatellites have been developed. This is largely due to the combination of its cost and researcher time efficiency, the ease of this non-species-specific method that combines the SNP discovery and screening steps, and the large number of SNPs reliably recovered.

The total approximate materials cost for our ddRAD-seq analyses, including DNA extraction, normalization of the DNA concentrations, library preparation, sequencing and computational time was US$3,270.00 for 240 samples, or approximately $13.63 per sample. The use of a homemade MagNA in place of commercial SPRI beads provides significant savings. This cost is similar to that for genotyping 240 individuals at 12 microsatellite loci (in 3 multiplexed PCR mixes), in a situation where the labelled primers have already been designed, purchased, and tested. However, a substantial additional

benefit of this ddRAD-seq method is that it does not require any locus discovery or development before starting. The time required for library preparation, once DNA has been extracted, is modest, and once the sequence data have been obtained, SNP calling for the entire dataset can be performed in less than a day through a largely automated bioinformatics pipeline. Unlike manually scoring peaks as in a traditional microsatellite genotyping analysis, the identification of SNPs is less subjective and takes far fewer hours of hands-on analysis (as most is performed computationally). The tools for analyzing these ddRAD data are freely available and widely used (e.g., Stacks, VCFtools).

For this study, our conditions and protocol allowed us to recover 411 high quality SNP loci for 240 individual samples. However, we show that through simple variations in the size selection window or the specificity of the restriction enzyme, more or fewer loci can be obtained. For some applications, it could be advantageous to multiplex a greater number of individuals and achieve similar coverage by aiming to recover fewer loci (e.g., using EcoRI rather than MspI). Alternatively, for applications where more loci are required, the size selection window could be widened and concordantly the number of individuals would have to be lowered.

The number of SNPs needed to perform robust parentage and relatedness analyses depends on characteristics of the study population. Populations with reduced genetic diversity will likely require a greater number of loci than those that are more genetically diverse (Saunders *et al.* 2007; Strucken *et al.* 2016; Tortereau *et al.* 2017). Obtaining more loci from the outset would aid in overcoming any issues relating to

population genetic diversity. Additionally, when studying species with complex social systems, including for example both variable levels of genetic relatedness among individuals and high rates of extra-pair fertilizations, it is imperative to obtain a sufficient number of markers to discern genetic relationships robustly (Hughes 1998; Ross 2001; Weinman *et al.* 2014). Our case study, using the variegated fairy-wren, shows that our modified ddRAD-seq method recovers more than enough SNP loci to confidently discern relationships in a species with a complex social system. Most parentage and relatedness analysis programs are well equipped to handle large numbers of loci, so a greater number of loci would not hinder analyses. Once an appropriate number of SNPs are identified for performing robust analyses, conditions can be varied to maximize the number of individuals to be genotyped.

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

482 the known mother was excluded from the paternity analysis, the microsatellite panel
483 sometimes produced assignments that were ambiguous (two males had similar LOD
484 scores), and occasionally the wrong male was assigned paternity of the offspring. Under
485 the SNP panel, ambiguous assignments were nonexistent, and these cases were clearly
486 resolved (Fig. 1).

487 It is sometimes difficult to obtain appropriate demographic data to use in a formal
488 parentage analysis, and for many studies, this level of detail may not be necessary.
489 Population allele frequencies can be used to estimate pairwise relatedness for
490 individuals, and to reconstruct pedigrees using maximum likelihood-based methods.
491 Variance in estimates of pairwise relatedness (*r*) for known parent-offspring pairs was
492 dramatically reduced when using SNPs (Fig. 3). For our simulations, SNPs greatly
493 improved the differentiation between distributions for individuals of known degrees of
494 relatedness (Fig. 4). This is particularly important for systems with minimal demographic
495 and observational data, where these distributions can be used to determine familial
496 relationships between individuals, in conjunction with actual estimated *r*-values.

497 In summary, our ddRAD-seq method provides a cost effective and robust way to
498 identify SNPs for use in studies utilizing parentage and relatedness analyses. Our
499 experiment shows that a majority of the same SNPs can be obtained across groups,
500 using the same size selection windows and restriction enzymes. Future individuals can
501 be genotyped and incorporated to the analysis by re-running the Stacks pipeline. Using
502 a bird exhibiting great social complexity, and high promiscuity, we have shown that
503 SNPs identified by ddRAD-seq are more effective at assigning paternity and estimating

relatedness than highly polymorphic, species-specific microsatellite loci. This protocol was designed to be universally applicable across bird species, and we have successfully applied it in a range of other avian study systems (Table 4). While different numbers of individuals were used in each study and therefore different numbers of loci were recovered, in all cases paternity was confidently assigned to nestlings using CERVUS (unpublished results). Applying this general protocol to many non-avian taxa may simply require ensuring that the specific restriction enzymes and fragment size windows are chosen appropriately.

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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.

# of samples (index groups)	enzymes	size selection interval
160 samples (8 index groups, index 1-8)	SbfI - MspI	450 - 600 bp
20 samples (Index 9)	SbfI - MspI	450 - 600 bp (replicate of above)
20 samples (index 10)	SbfI - MspI	400 - 700 bp (wide size selection)
20 samples (index 11)	SbfI - EcoRI	450 - 600 bp (infrequent 3' cutter)
20 samples (index 12)	SbfI - EcoRI	450 - 600 bp (replicate of above)

Table 2. Marker characteristics

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}

*Only biallelic SNPs were retained. If a locus had 3 alleles across the population, it was filtered from the dataset.

769 Table 3. Overlap in the RAD loci that were obtained while varying different steps of the
770 protocol (size selection and restriction enzymes). The diagonal indicates the total
771 number of loci recovered for each treatment. Values above the diagonal represent the
772 percent overlapping loci between groups (relative to the group with the smallest number
773 of loci), while values below the diagonal list the number of loci that were overlapping
774 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

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All: 160 samples; Sbf1/Msp1; 450-600 bp.
A: 20 samples; Sbf1/Msp1; 450-600 bp.
B: 20 samples; Sbf1/Msp1; 450-600 bp.
C: 20 samples; Sbf1/Msp1; 400-700 bp.
D: 20 samples; SbfI/EcoRI; 450-600 bp.
E: 20 samples; SbfI/EcoRI; 450-600 bp.

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777 Table 4: Summary information for ddRAD-seq studies performed to investigate
778 parentage in other bird species.

Species	Scientific name	Number of individuals	Number of loci	Number of loci in HWE
Variegated fairy-wren	*Malurus lamberti*	160	552	411
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*	240	483	233
		240	291	174

779 * Two independent ddRAD-seq experiments were performed - one with 240 samples
780 and the other with 48. After quality filtering and demultiplexing the data from 288
781 samples was combined for denovo assembly and SNP identification.
782 ** Two independent ddRAD-seq experiments were run on each set of 240 samples.
783 There is 74% overlap in the loci identified in each experiment (of the loci in HWE).
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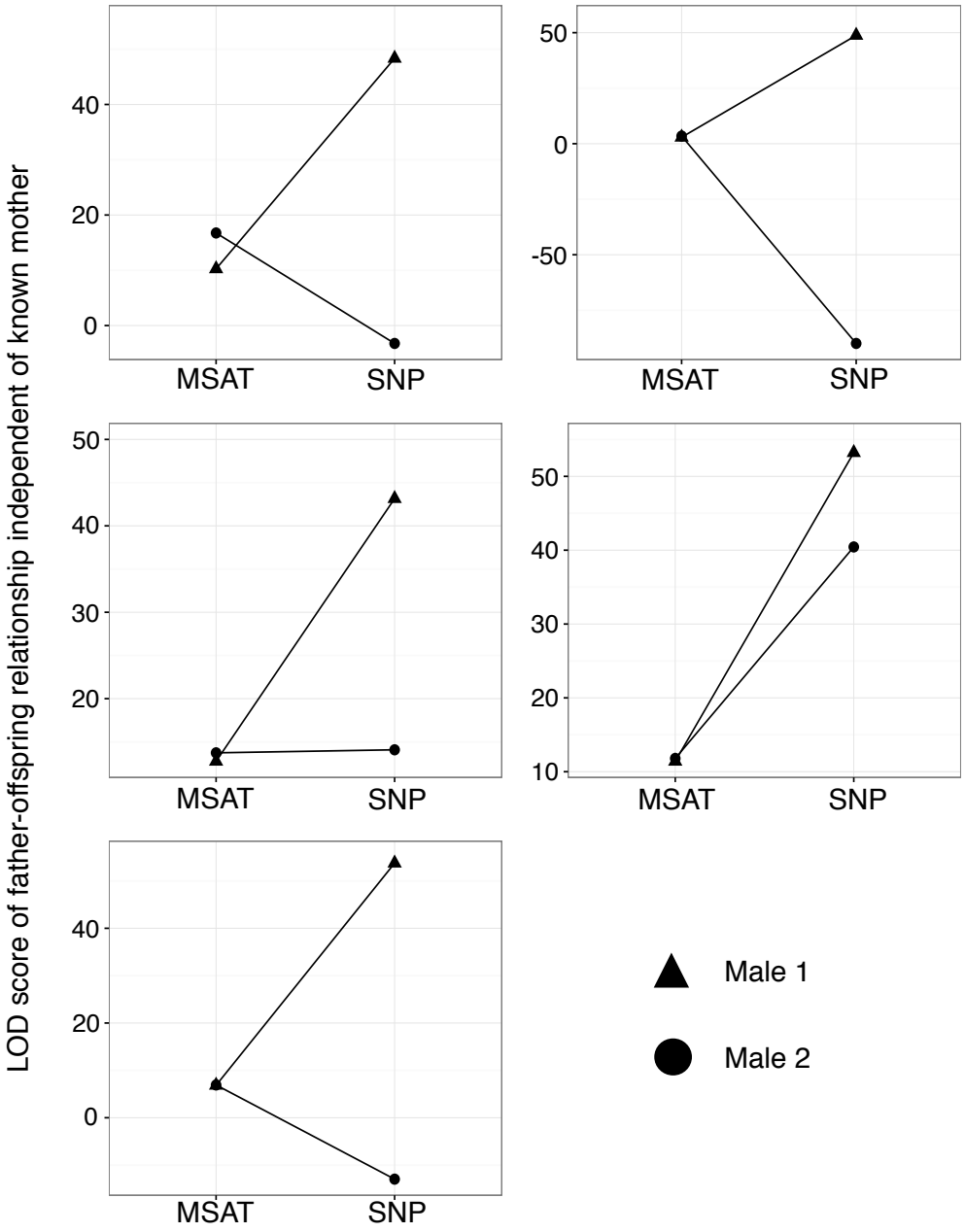
Figures

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 y-axis scale varies among panels in the graph.



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

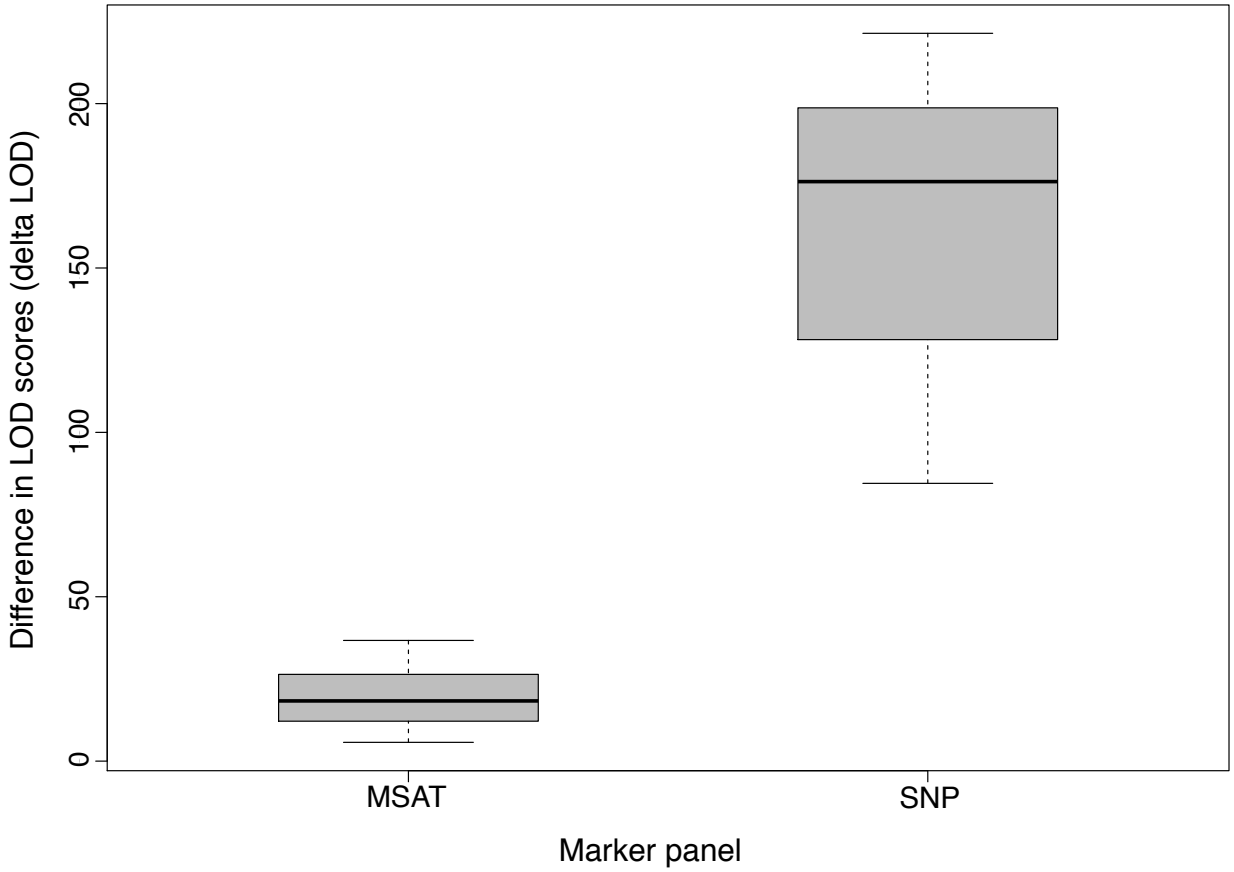


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Figure 3. Box plot of pairwise relatedness values for all parent-offspring (40 mother-offspring and 40 father-offspring) relationships, using population allele frequencies from each marker panel.

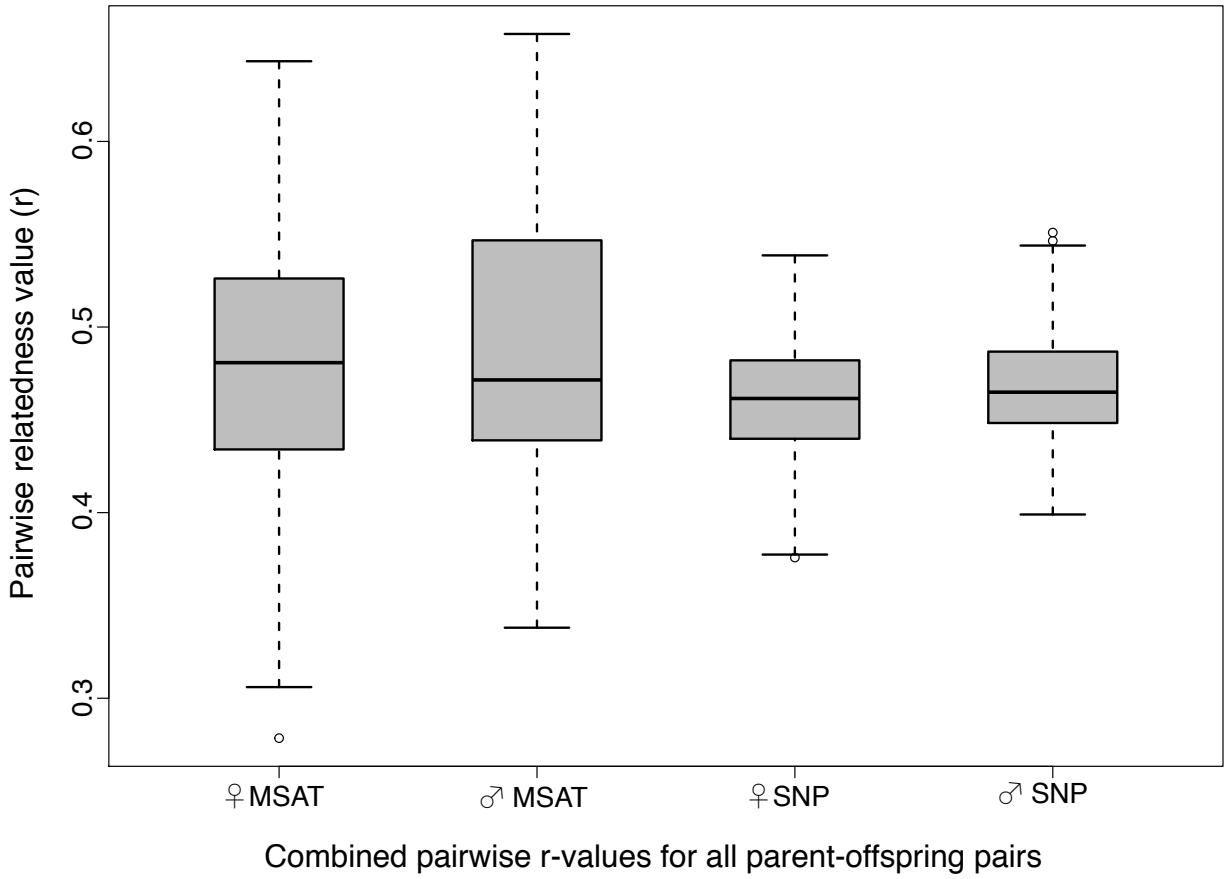

Figure 4. Density plots of relatedness values for simulated pairs of known relatedness (unrelated, half-sibling, full-sibling, and parent-offspring) using population allele frequencies from each marker panel (a. MSAT; b. SNP). Overlap in distributions indicates the overlap between relatedness value estimators 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).