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1 **Performance and precision of double digestion RAD (ddRAD) genotyping in large**
2 **multiplexed datasets of marine fish species**

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35 **Abstract**

36 The development of Genotyping-By-Sequencing (GBS) technologies enables cost-effective
37 analysis of large numbers of Single Nucleotide Polymorphisms (SNPs), especially in ‘non-
38 model’ species. Nevertheless, as such technologies enter a mature phase, biases and errors
39 inherent to GBS are becoming evident. Here, we evaluated the performance of double digest
40 Restriction enzyme Associated DNA (ddRAD) sequencing in SNP genotyping studies
41 including high number of samples. Datasets of sequence data were generated from three marine
42 teleost species (>5,500 samples, >2.5x10¹² bases in total), using a standardized protocol. A
43 common bioinformatics pipeline based on STACKS was established, with and without the use
44 of a reference genome. We performed analyses throughout the production and analysis of
45 ddRAD data in order to explore (i) the loss of information due to heterogeneous raw read
46 number across samples; (ii) the discrepancy between expected and observed tag length and

47 coverage; (iii) the performances of reference based vs. *de novo* approaches; (iv) the sources of
48 potential genotyping errors of the library preparation/bioinformatics protocol, by comparing
49 technical replicates. Our results showed use of a reference genome and *a posteriori* genotype
50 correction improved genotyping precision. Individual read coverage was a key variable for
51 reproducibility; variance in sequencing depth between loci in the same individual was also
52 identified as an important factor and found to correlate to tag length. A comparison of
53 downstream analysis carried out with ddRAD vs single SNP allele specific assay genotypes
54 provided information about the levels of genotyping imprecision that can have a significant
55 impact on allele frequency estimations and population assignment. The results and insights
56 presented here will help to select and improve approaches to the analysis of large datasets based
57 on RAD-like methodologies.

58 **Introduction**

59 The options for studying the genomic constitution of individuals and populations are increasing
60 rapidly thanks to the development of powerful and accurate sequencing technologies that
61 provide higher throughput at decreasing costs (Liu et al. 2012). Meanwhile, efficient reduced
62 representation methods have been proposed to provide high sequence coverage for selected
63 genomic regions, collectively named as Genotyping-By-Sequencing (GBS) technologies
64 (Narum et al. 2013). One of these GBS methods, Restriction-site Associated DNA sequencing
65 (RAD-seq) (Baird et al. 2008) has become particularly popular as it allows the cost-effective
66 analysis of thousands of markers for tens/hundreds of individuals in a single sequencing lane.
67 The original RAD protocol has also been modified to optimize throughput and ease of use,
68 generating several alternative RAD-like methods (*e.g.* Peterson et al. 2012; Wang et al. 2012;
69 and the review by Andrews et al. 2016).

70 As GBS technologies enter a more mature phase, biases and errors inherent to such methods
71 are becoming apparent (Arnold et al. 2013) and comparative analysis of the most popular RAD-
72 like protocols have addressed some of these subjects (Puritz et al. 2014). Two recent studies
73 (DaCosta e Sorenson 2014; Mastretta-Yanes et al. 2015) focused specifically on genotyping
74 issues relating to double digest Restriction enzyme Associated DNA (ddRAD) (Peterson et al.
75 2012). ddRAD is one of the most recently developed RAD variants, known for its relative
76 flexibility and ease of use. In addition to the sources of error that also affect other
77 methodologies, the authors recorded ddRAD-specific issues such as the recovery of restriction
78 fragments shorter than expected, amplification bias toward GC-rich fragments, non-specific
79 cutting by restriction enzymes, newly formed restriction enzyme sites and drop of fragment
80 number due to loss of restriction sites.

81 Beyond laboratory-based assessments of variation in ddRAD performance, there is a need to
82 better understand the risk of errors associated with the production and use of ddRAD data,
83 which is becoming increasingly relied upon for population genetic inference. Unawareness of
84 the presence of biased markers can indeed lead to artificial excess of homozygotes (Taberlet et
85 al. 1996), false departure from Hardy–Weinberg equilibrium (Xu et al. 2002), overestimation
86 of inbreeding (Gomes et al. 1999) and unreliable inferences about population structure that have
87 the potential to distort research conclusions. As a consequence, natural resource management
88 and policy can be seriously affected. In this study, we seek to expand the experimental
89 evaluation of ddRAD by focusing on the performance of common bioinformatics approaches
90 as applied to multiple, comparable, large ddRAD datasets of marine fish species. A technical
91 evaluation focused on marine fish data is interesting due to some biological characteristics of
92 this taxon, such as relatively high SNP frequency, that can further affect genotyping accuracy.
93 The species analyzed in this study are the European sea bass (*Dicentrarchus labrax*), the
94 gilthead sea bream (*Sparus aurata*) and the turbot (*Scophthalmus maximus*).

95 Available genomic resources are increasing for three species studied. Sea bass (Tine et al. 2014)
96 and turbot (Figueras et al. 2016) genomes have already been published and a draft sea bream
97 genome will soon be published (L. Bargelloni, personal communication) and was made
98 available for this work. The three differ in the quality of their assembly, as indicated by the
99 contig length (i.e. their respective N50 values, which is defined as the length N for which 50%
100 of all bases in the sequences are in a sequence of length $L < N$). However, they share similar
101 genome size and can thus provide comparable results (Table 1). The use of species with
102 different levels of genome sequence development permits assessing effects of the reference
103 genome quality on approaches that use genomes to improve the performance of clustering

104 methods for RAD data (e.g. reference based analysis in STACKS).

105 In this study, we set out to examine how variation in ddRAD sequence datasets and the
106 application and quality of available reference genome sequences affect the consistency and
107 accuracy of resulting data, generated through commonly used analytical approaches. The
108 laboratory and bioinformatic pipeline used to generate the ddRAD datasets followed standard
109 published methods (see below) and has been summarized in a flowchart (Figure 1). The
110 performance of the ddRAD pipeline was evaluated at different stages in order to investigate the
111 causes and effects of variation in individual sample coverage, RAD-tag sequence length and
112 application and quality of reference genomes on the eventual accuracy and error rates of
113 individual genotyping. We specifically addressed the following questions:

114 (i) *Evaluation of sample representation within multiplexed libraries.* What is the
115 typical variation in terms of number of raw reads per sample when multiple
116 individuals (144 in our case) are multiplexed in a single sequencing lane?

117 (ii) *Tag length and coverage.* Is there any difference between the expected and observed
118 length of analyzed tags? Does any relationship exist between tag length and depth
119 of coverage?

120 (i) *De novo and reference-based genotyping using STACKS.* What is the effect of
121 different clustering approaches (e.g. *de novo* vs reference-based, *a posteriori*
122 genotyping correction) on the number of markers identified?

123 (ii) *Genotyping precision and error rates.* What are the effects of the variables described
124 above on the number of mismatches between technical replicates?

125 Based on these insights we suggest approaches which can help to mitigate the identified risks
126 of error in ddRAD analysis. Finally, the potential effect of genotyping imprecision on

127 downstream analysis was evaluated using comparative data between ddRAD and single SNP
128 allele specific genotyping, focusing on how genotyping errors could affect allele frequency and
129 population assignment.

130 **Material and Methods**

131 *Samples and library preparation*

132 Specimens of European sea bass, gilthead sea bream and turbot were collected in the context of
133 the European Union's FP7 funded project 'AQUATRACE' (KBBE 311920). The entire sample
134 set included more than 5,581 specimens (2,128 European sea bass, 2,156 gilthead sea bream
135 and 1,297 turbot) from the species' distribution range, some of which were collected
136 specifically for the project (years 2013-2014, from now on referred to as "fresh" samples), while
137 others had been collected earlier ("archived" samples) (Supplementary Material Table). For
138 fresh samples, fin clips were preserved separately in 95% ethanol at 4°C until genomic DNA
139 (gDNA) extraction. Samples were extracted either with Invisorb® DNA tissue HTS 96 kit
140 (Strattec biomedical) or with a standard NaCl isopropanol precipitation protocol (Cruz et al.
141 2016). Extracted DNA samples were then classified as "high", "mid" or "low" quality
142 according to the level of degradation assessed with agarose gel electrophoresis (see
143 Supplementary material).

144 The same ddRAD protocol, with minor modifications, was used for the three species. The
145 library preparation followed the original guidelines of Peterson et al. (2012), with some
146 modifications that facilitate the screening of large number of individuals (see Supplementary
147 Material for details), and was carried out in three different laboratories within the AquaTrace
148 consortium, each focusing on a single species: the sea bass at the Laboratory of Biodiversity
149 and Evolutionary Genomics, University of Leuven, sea bream at the Department of Compared

150 Biomedicine and Food Science, University of Padova and turbot at the Departamento de
151 Zoología, Genética y Antropología Física, Universidade de Santiago de Compostela. To
152 promote a common standardized approach, staff from the three laboratories completed a hands-
153 on training course in library preparation at the Institute of Aquaculture, Stirling, where the
154 modified ddRAD protocol originated. Multiple ddRAD libraries were prepared for each species
155 (sea bream n=14; sea bass n=14; turbot n=9). Each library comprised 144 samples, and in all
156 the libraries the same three or four control samples for each species were included, to enable
157 cross-library comparisons and mismatch rates between replicates to be assessed. In particular,
158 four sea bream specimens (SAC3, SAC4, SAC5 and SAC6 from Sardinia, Italy); three sea bass
159 specimens (DLTY40, from the Central Mediterranean Sea; DLM44, from the Atlantic and
160 DLFF1, from a European broodstock); and four turbot specimens (SMFF1, SMFF2 and SMFF3
161 from a Spanish broodstock; SMNS32 from North Sea's wild population) were used.

162 *Sequence data analysis – standard pipeline*

163 The following approach to sequence data analysis was used for all datasets as the basis for
164 subsequent comparative analysis. Raw data were filtered to retain only high quality reads, using
165 STACKS 1.28 (J. Catchen et al. 2013; J. M. Catchen et al. 2011) *process_radtags* program,
166 which allows simultaneous quality filtering and sample demultiplexing. After barcode removal
167 (5-7 bases), the sequences were 3' end-trimmed to a standard 90 nucleotides length. Each read
168 was then analyzed to assess sequence quality. Briefly, a 3-base sliding window (STACKS'
169 option *-w*) was used to parse each read and where the average phred score of three consecutive
170 bases was lower than 20 (STACKS' option *-s*) the entire read was discarded.

171 STACKS was also used for clustering reads and for SNP discovery, following standard *de novo*
172 and reference based pipelines, well described in the program website

173 (<http://catchenlab.life.illinois.edu/stacks/>). In our case parameter $-m$ (minimum number of
174 reads to call a stacks) was set to four and $-M$ (maximum number of mismatches between reads
175 to be considered as part of the same cluster) was set to five, according to the suggestion of
176 Mastretta-Yanes (2015), and considering the longer reads of our study (due to concatenation).
177 For the *de novo* approach, reads from primer P1 were concatenated with the reverse
178 complement sequence of reads from primer P2, obtaining 180 bp *pseudo-contigs*. This approach
179 was used to create longer sequence tags which reduces the risk of over-merging (i.e. clustering
180 together tags coming from different genomic regions) by keeping the information about relative
181 proximity of Read 1 and Read 2. As an added benefit, this approach allowed to be fully aware
182 of linkage issues. Since reference based approach require reads to be mapped against a
183 reference, we used the software package BOWTIE (Langmead et al. 2009), considering read
184 pairing in the alignment process. We kept only read pairs that matched a single genomic
185 position.

186 When building the RAD-tag catalog a maximum number of five mismatches between tags was
187 set. For the reference-based approach, clustering was based on mapping position. Within a stack
188 containing variant reads (i.e. potential SNPs), the following call thresholds were used: rare
189 variant frequency (rvf) <0.01 = homozygote; $0.01 < \text{rvf} < 0.1$ = 'genotype unknown'; $\text{rvf} > 0.1$ =
190 heterozygote called. *rxstacks*, STACKS' component that corrects genotypes on the basis of
191 population information, was also implemented for comparison. Finally, we used the algorithm
192 implemented in STACKS' *populations* step to retain only individual loci represented with at
193 least 10 reads per individual sample and genotyped in at least 80% of the samples analyzed.
194 This is an important step when the genotypes of multiple individuals need to be compared, as
195 only shared loci provide useful information for genetic analysis.

196 *Analysis of the pipeline*

197 Here, we describe the methods used to assess the pipeline based on the four issues described in
198 the Introduction (Figure 1).

199 (i) *Evaluation of sample representation within multiplexed libraries*

200 Considering the number of samples multiplexed and the average output of the sequencing
201 platform/chemistry (180 M reads), approximately 1.3 M reads per sample are theoretically
202 expected. However, even if initial DNA quantification is accurate and input DNA is equal
203 among samples, subsequent library preparation steps may alter individual representation within
204 the library resulting in variability in inter-sample sequencing effort. To investigate sample read
205 homogeneity in libraries with up to 144 pooled individuals, we first established a threshold
206 number of reads per sample against which to filter individual sample data. A threshold of 150
207 k reads was chosen as a minimum to accept an individual sample for downstream data
208 processing, based on an expected number of 7,000 stacks per sample (estimated from *in-silico*
209 analysis) and an average coverage of 20x. This threshold was used in the analysis of the
210 sequencing output for all available ddRAD data including more than 5,000 samples.

211 To identify the factors correlated with fewer reads, we tested the correlation between number
212 of reads (above or below the threshold) and variables such as “DNA quality”, whether a sample
213 was “fresh” or “archived”, “individual sample collector” (i.e. the project partner that collected
214 the sample), and “index barcode” (different length/sequence barcodes could perform differently
215 in the amplification or sequencing by synthesis steps), testing the effect of each variable under
216 a Generalized Linear Model (GLM), as implemented in R 3.2.3 library function Rcmdr (Team
217 2013; Fox 2005). Chi-squared tests were applied to check association between tested variables.

218 For the analysis described further on, only replicate samples with sufficient read numbers were
219 used.

220 (ii) *Tag length and coverage*

221 To understand whether the length of the RAD-tags ('tag length') observed corresponded to the
222 expected length (i.e. the 'insert length' from size selection minus adapter length) and to
223 investigate association between tag length and coverage, we extracted fragment length and
224 DNA sequences of ddRAD-tags from BOWTIE alignment results. Data on coverage depth was
225 extracted for each single locus of each sample, separately. To allow comparison between
226 samples with different average coverage, standardized coverage depth was obtained by dividing
227 locus specific values by the average coverage across all loci for each sample. Similarly, when
228 comparing the distribution of the number of tags with different lengths, 10 bp bins were used
229 and the relative number of tags was calculated dividing the number of tags of a certain length
230 bin by the average number of tags across all the bins. A Wilcoxon signed-rank test, as
231 implemented in R 3.2.3 library Rcmdr (Team 2013; Fox 2005), was used to test for differences
232 between distributions from the three datasets.

233 (iii) *De novo and reference-based genotyping using STACKS*

234 In order to understand how the alignment to a reference genome influences SNP genotyping,
235 we obtained individual genotypes using both *de novo* and reference-based analysis in STACKS.
236 Since we expected *de novo* approach to detect also tags that are not contained in the reference
237 genome, we wanted to evaluate the amount of *de novo* tags that could be found in the genome.
238 In order to do this, RAD-tags resulting from *de novo* analysis (180 bp long) were subsequently
239 split in two (in order to reconstitute the original 90 bp tags) and mapped against the reference
240 genome using BOWTIE, with the same parameters used while aligning reads for reference based

241 analysis. Under both *de novo* and reference-based analysis, results were compared with and
242 without the final step in *rxstacks*. Statistical differences between approaches were tested with
243 chi-squared tests.

244 (iv) *Genotyping precision and error rates*

245 To investigate the level of reproducibility across different bioinformatic approaches we
246 examined the level of consistency among scored SNP genotypes within the sets of nine to 15
247 replicated samples for each species. The most frequent genotypes were considered as the
248 “correct” ones, and mismatches were counted for each locus in each sample to estimate
249 genotyping error.

250 When comparing results from different approaches, statistical significance was tested using
251 either on-line applications (e.g. Kruskal-Wallis: <http://vassarstats.net>) or the Rcmdr library for
252 R 3.2.3 (Team 2013; Fox 2005). A first global analysis was carried out to assess the effect of
253 several parameters (“coverage”, “genome reference” mapping, “*rxstacks* correction”,
254 percentage of high-quality reads) on mismatch rate across the entire dataset. Individual
255 mismatch rates were classified either as a binary outcome (0 for values lower than the overall
256 median mismatch rate, 1 for those equal or greater), or grouped into quartiles for a finer
257 evaluation of the effects of different explanatory factors. In both cases, either a Generalized
258 Linear Model (used with binary outcome) or Ordinal Linear Regression (used with samples
259 grouped into quartiles) were used to detect the most influential variables. The same statistical
260 approach was then implemented, within each dataset, across single specimens, to look more
261 into detail at individual-specific features that could affect genotyping quality and to avoid
262 dataset-specific biases and errors. This additional analysis was possible thanks to the large
263 number of replicates available for each species and the standardization of library preparation

264 technique and bioinformatics protocols. Lastly, mismatch rates were analyzed across loci, to
265 check the expectation that, within each “species+strategy” dataset (e.g. sea bream+*de novo* or
266 turbot+reference based) loci with lower average coverage also showed higher mismatch rates.

267 *Assessing the impact of genotyping accuracy*

268 The impact of genotyping imprecisions on downstream applications varies depending on the
269 type of analysis carried out. Since the principal applications of the project data were the analysis
270 of genetic structure, based on allele frequency, and the development of traceability tools, based
271 on population assignment, we evaluated the impact of variation in genotype scoring on these
272 applications. To do this, we used a reduced set of highly informative markers (14 for sea bass,
273 15 for sea bream and 18 for turbot) genotyped with both ddRAD and with a single SNP allele-
274 specific assay (KASP). Using these approaches we genotyped 22, 25 and 22 samples of sea
275 bass, sea bream and turbot, respectively. Comparison of the two genotype datasets was
276 conducted at the following three levels: a) *Genotype data*: a simple analysis of genotype
277 mismatch between data from the two approaches analysed in the same individual fish, with missing
278 data differentiated from observed differences in genotype; b) *Allele frequency data*: the impact of
279 individual genotyping mismatches on allele frequencies was assessed by testing for statistical
280 significance (Student’s T-tests) between allele frequencies across all loci, with genotype
281 differences not differentiated from missing data in their effect on allele frequencies; c) *Individual*
282 *assignment data*: assignment was conducted using GeneClass2 software (Piry et al. 2000).
283 Individual assignment scores (%) output from GeneClass2 were used to assess differences in
284 assignment of individual genotypes produced using the two methods and significance was assessed
285 using Student's T-tests. Reference data for population assignment consisted of a larger set of more
286 than 900 wild and farmed samples for the three species genotyped with ddRAD.

287

288 **Results**

289 The first part of the study addressed the loss of analytical power in terms of number of samples
290 filtered due to unequal representation of individuals within libraries; it was based on a data set
291 of more than 5,581 samples, in which the replicate individuals were included.

292 (i) *Evaluation of sample representation within multiplexed libraries*

293 As indicated by high values of standard deviation (in particular for turbot), variation in the
294 number of raw reads among individuals within species was very high. In fact, 129 samples (71
295 sea bass, 16 sea bream and 42 turbot) were represented by less than 1,000 reads and three
296 samples (all in turbot dataset) had more than 5,000,000 reads. Using the threshold of 150,000
297 raw reads, 6.8% of sea bass samples, 8.1% of sea bream samples and 16.0% of turbot samples
298 were discarded. After quality filter was applied, an average of $74.5\% \pm 10.8\%$ reads remained
299 available for further analysis. After filtering, the average number of high quality reads was
300 similar across species, $687,426 \pm 447,701$ in European sea bass, $614,099 \pm 406,018$ in gilthead
301 sea bream and $610,703 \pm 707,152$ in turbot. Regression analysis indicated that better quality
302 DNA resulted in higher number of high quality reads ($t = -11.4$ $p < 0.001$); similarly, “fresh”
303 samples had a higher amount of high quality reads than “archived samples” ($t = -3.1$ $p < 0.005$).
304 “DNA quality” of individual samples was neither significantly associated with species ($X^2 = 4.6$
305 $p > 0.25$), nor with fresh/archived condition ($X^2 = 3.1$ $p > 0.25$). The DNA of 129 samples showing
306 less than 1,000 reads were all of good quality, which means that inaccurate quantification or
307 pipetting errors are probably what caused this strong under-representation.

308 After filtering and quality checking, the final number of replicated samples available for
309 downstream analysis was 111: 43 sea bream samples (11 replicates for SAC3, 11 for SAC4, 10

310 for SAC5 and 11 for SAC6) genotyped across 11 independent libraries, 34 sea bass samples (5
311 replicates for DLCTY_40, 14 for DLT_1 and 15 for DLM_44) genotyped across 15 libraries
312 and 34 turbot samples (9 replicates for SMFF1, 8 for SMFF2, 9 for SMFF3 and 8 for SMNS32)
313 genotyped across 9 libraries.

314 (ii) *Tag length and coverage*

315 On average across species, 78.4% of the reads were successfully mapped on the reference
316 genomes and mapping rates ranged from 71.3% uniquely mapped reads in sea bream to 85.4%
317 in sea bass.

318 Average tag length across datasets was 288.9 ± 110.5 bp. Most of the tags (79.5%) were 100-
319 380 bp. In addition, substantial fractions (21.1% sea bream, 24.5% sea bass, 15.9% turbot) of
320 analyzed RAD-tags were shorter than 190 bp (the minimum size expected according to the
321 library construction protocol) (Figure 2). Paired-tests between datasets suggested that size
322 distribution was not significantly different across species (Wilcoxon signed-rank test, bream-
323 bass $p=0.803$, bream-turbot $p=0.865$, bass-turbot $p=0.984$).

324 Although average coverage depth per locus differed among datasets for the three species (157
325 ± 94 for sea bass, 248 ± 126 for sea bream, 700 ± 544 for turbot), relative coverage was evenly
326 distributed (Wilcoxon signed-rank test, bream-bass $p=0.697$, bream-turbot $p=0.865$, bass-turbot
327 $p=0.689$) with respect to RAD-tag length (Figure 3). Significant ($p<0.01$) positive linear
328 correlations between length and coverage were also found for fragments in the range from 100
329 to 250 bp (Spearman $\rho=0.903$ in sea bream, 0.957 in sea bass and 0.918 in turbot). Fragments
330 longer than 250 bp showed significant ($p<0.01$) negative linear correlation between length and
331 coverage (Spearman $\rho=-0.969$ in sea bream; -0.968 in sea bass, -0.952 in turbot). No
332 significant correlation between GC content of fragments and coverage depth was observed.

333 (iii) *De novo and reference-based genotyping using STACKS*

334 The number of independent RAD-tags identified varied depending on the approach. In all cases
335 the number of tags found by the reference genome-based approach was much lower than that
336 found with the *de novo* approach (up to 5.5 times, in turbot dataset) (Table 2). However, when
337 a filter was applied to retain only tags shared by at least 80% of samples analyzed, higher
338 proportion was retained for reference-based analysis (on average $44.9\% \pm 19.7\%$) than *de novo*
339 analysis (on average $9.1\% \pm 6.0\%$). This made that in most cases the final number of retained
340 tags was higher using the reference-based approach. Similarly, a higher number of SNPs was
341 observed in the reference-based approach after filtering. The application of the genotype
342 correction implemented in *rxstacks* reduced the number of tags by different extents: a minimum
343 of 63% of total tags were retained in the turbot reference-based analysis and a maximum of
344 99.6% in the sea bass *de novo* analysis. The proportion of SNPs retained was comparable,
345 ranging from 56.9% to 99.8% in turbot (reference-based) and sea bass (*de novo*), respectively.
346 Mapping tags from *de novo* analysis against the reference genomes produced 11,121 matches
347 for sea bass (28.3% of *de novo* RAD tags); 11,650 for sea bream (23.0% of *de novo* RAD tags)
348 and 7,889 for turbot (6.8% of *de novo* RAD tags). These figures are in agreement with the
349 relative length of the genomes utilized (Table 1), while the lower than expected difference
350 between sea bass and sea bream results can be explained by the lower quality of the bream
351 assembly, as indicated by the N50 value.

352 (iv) *Genotyping precision and error rates*

353 Our analysis suggested that “*rxstacks* correction” and “coverage” significantly affected the
354 level of accuracy in the comparison of different approaches, regardless the species. In particular,
355 lower mismatch rate were recorded when *rxstacks* was implemented and when coverage depth

356 per sample was higher. However, variation in mismatch rates were found between different
357 species datasets (Table 3); they were apparently linked with differences in species-specific
358 coverage, which varied significantly both for *de novo* RAD-tags (Kruskall-Wallis test, $H=15.27$
359 $p<0.001$) and reference-based ones (Kruskall-Wallis test, $H=30.74$ $p<0.0001$). To overcome
360 biases linked to species-specific differences, more specific tests were carried out within single
361 datasets. In fact, additional factors were found to be significantly affecting mismatch rate. In
362 addition to “*rxstacks* correction”, also “library”, “reference-mapping” and “sample” (only in
363 the turbot database) showed significant correlations. At species level, “Coverage” showed a
364 significant correlation in two out of three datasets (sea bream ($p<0.05$) and turbot ($p<0.001$)).
365 Nevertheless, across loci (i.e. within “single species+strategy” dataset) no significant
366 correlation between mismatch and coverage was found.

367 *Impact of genotyping accuracy on population assignment*

368 The percentage of samples with at least one different genotype observed in sea bass, sea bream
369 and turbot was 31.5% (seven samples), 52% (13 samples) and 36% (eight samples), respectively,
370 reflecting a total level of genotyping variation of 3.7% in sea bass (nine allelic differences), 4.8%
371 in sea bream (eighteen allelic differences) and 3.0% in turbot (12 allelic differences). Resulting
372 allele frequencies differed significantly at one locus in sea bream, that displayed the largest single
373 allele frequency difference between the two genotype datasets (11.5%). A similar single locus
374 deviation was observed in sea bass, despite the overall difference being non-significant. The effect
375 of genotyping mismatch on individual assignment was lower; only for turbot, was one individual
376 assigned to different populations of origin with the two genotype datasets. On closer inspection,
377 the two genotypes for this sample differed at three alleles observed at two loci. The two genotypes
378 at first locus were alternate homozygotes, whereas the other discrepancy was between

379 heterozygous and homozygous genotypes at another locus. Further analysis of the sample based
380 on population exclusion testing revealed that neither population of origin (wild or farmed) was
381 excluded using the two genotype datasets meaning that misassignment would be possible in this
382 scenario. When this individual was excluded from the sample dataset, there was no significant
383 difference in quantitative assignment scores between genotypes from RAD and Kasp approaches.
384 Neither sea bream nor sea bass exhibited discrepancies in population assignment, nor in
385 assignment scores, obtained with different sets of genotype data.

386 **Discussion**

387 The aim of the present work was to quantify the level of genetic information that can be obtained
388 with ddRAD approach, net of information loss during bioinformatic processing; and to evaluate
389 the performance of different bioinformatics approaches on the number of markers detected and
390 the precision of the genotype calling. The use of large datasets of marine fish species and the
391 application of the same approaches as those used in real case studies make our results
392 informative on the practical application of this technique.

393 (i) *Evaluation of sample representation within multiplexed libraries*

394 The first step in which genotyping information is lost is quality filtering, required to obtain
395 reliable results with NGS analysis (Minoche, Dohm, and Himmelbauer 2011; Bokulich et al.
396 2013). The filtering used in this work was stricter than the default of STACKS filtering, and
397 probably stricter than many of the filtering approaches used in population genetics studies.
398 However in this study our filtering process did not result in the loss of many samples; where
399 samples were removed this was mostly due to low initial read depth resulting from unequal
400 sequencing effort.

401

402 Relaxing the STACKS filtering parameters would be one method of retaining more reads,
403 however this would risk increasing genotyping error. An alternative approach, given that low
404 sequence quality is typically concentrated at the read ends, would be to employ further trimming
405 of all reads prior to commencing STACKS analysis. This should have the effect of retaining
406 more reads during subsequent STACKS filtering based on phred-scores. Nevertheless, this
407 procedure still causes loss of potentially high quality genetic information and a more efficient
408 approach would be to trim only those reads affected by low quality instead of trimming every
409 read to the same extent. This would only be possible if the downstream SNP caller program
410 allowed for different length reads (unlike STACKS).

411 One of the main advantages of RAD techniques is the possibility of multiplexing many
412 individuals in the same sequencing run thanks to individual sample barcoding. However, as the
413 number of multiplexed individual samples increases, the chance to have poorly represented
414 samples increases as well (Baird et al. 2008; Peterson et al. 2012), causing lower coverage and
415 in the worst case, too few reliably genotyped or false homozygote excess for a number of
416 individuals. In particular, the combination of samples at different quality/concentration, rather
417 than the quality itself of single samples, is the influencing variable (I.e. even using the exact
418 same starting DNA, result might vary in relation with the other samples genotyped in the same
419 library). The threshold at 150,000 raw reads used here is much lower than the expected average
420 number of reads per individual (1.3 millions) and may not be appropriate for other species. In
421 fact, it should be set taking into consideration the number of expected tag and the desired
422 average coverage depth. However, “losing” a certain amount of samples (up to 16% in our case)
423 needs to be considered when planning a ddRAD sequencing project, even when significant
424 effort was given to equalize DNA input under library preparation.

425 Not surprisingly, DNA quality was a good predictor of poorly performing samples (Graham et
426 al. 2015). Gel-based quality analysis essentially reflects the level of DNA degradation, that can
427 be caused by many factors that act before or after extraction. In our specific case, pre-extraction
428 factors are probably the most relevant, as extraction and post-extraction protocols were the same
429 for all the samples. Ethanol has been recognized as a good media for long term tissue storage
430 (Gillespie et al. 2002; Dawson, Raskoff, and Jacobs 1998), and it is easily available and not
431 hazardous. Nevertheless, Seutin, White, and Boag (1991) reported that ethanol conservation
432 can decrease DNA yield and cause significant degradation to the extracted DNA, that can be
433 reduced by keeping samples refrigerated as soon as possible after sampling. DNA from long-
434 term stored specimens might have some additional features reducing the efficiency in library
435 preparation. Therefore, when selecting the DNA samples to be pooled as part of the same
436 library, it is advisable to avoid mixing samples of heterogeneous DNA quality as well as mixing
437 “fresh” with “archived” specimens. When this is not possible (e.g. for those projects that use
438 only one or few sequencing pools), an upward correction for the starting amount of DNA of
439 poor quality samples and DNA from “archived” samples might be considered. However, further
440 analysis is necessary to better understand how this procedure should be applied.

441 (ii) *Tag length and coverage*

442 Accuracy and consistency in size selection is not easily achievable, but tag length distribution
443 was not significantly different across species in our study. From this point of view, the period
444 of training of the personnel proved to be effective in order to have consistent results.
445 Nevertheless, tags shorter than 190 bp were retained in our analysis, which was unexpected
446 considering that size selection step was implemented. Indeed, low accuracy has been
447 documented in particular for manual vs automated gel band extraction (Puritz 2015). A similar

448 result was found by DaCosta e Sorenson (2014), who recovered tags down to a length of 10 bp.
449 In our case, the purification steps performed at the very end of the library preparation protocol,
450 should eliminate most inserts shorter than 200 bp, that translates into RAD tags of 75 bp, after
451 removing adapters. It is important to notice that, considering the 100 bp paired-end sequencing
452 protocol used, all the analyzed fragments shorter than 190 bp are affected by Read1-Read2
453 overlapping of the final parts of the sequences, potentially causing SNP duplication, redundant
454 data and a waste of sequencing effort that further lower the actual power of ddRAD technique.
455 Improvement in size selection step is fundamental to optimize the performance of the ddRAD
456 technique.

457 Davey et al. (2013), using data from a *Caenorhabditis elegans* RAD library, found a strong
458 positive correlation between fragment length and coverage depth. In other published ddRAD
459 studies, such as DaCosta e Sorenson (2014), the relationship between coverage and length was
460 similar to our work. Tags with different lengths show variable coverage within individual
461 samples. This means that additional care should be taken when multiplex size is calculated, in
462 order to achieve a desired minimum depth of coverage across loci. According to our results,
463 loci in the shortest and longest length range will be underrepresented if coverage was calculated
464 just by dividing the number of individual reads by the number of expected loci. Upward
465 correction in the number of reads per individual should be applied to obtain minimum coverage
466 also for loci in short and long fragments.

467 (iii) *De novo and reference-based genotyping using STACKS*

468 The possibility to use RAD techniques in species without genomic resources (i.e. *de novo*
469 approach) has been highlighted as one of the method's biggest advantages (Willing et al. 2011;
470 Pegadaraju et al. 2013). However, we showed that using a reference genome improves RAD

471 genotyping performance, i.e. better precision and higher number of shared markers. With
472 reference based approach, only reads correctly mapped against the genome are used. Hence, the
473 quality of reference-based analysis is also dependent on the quality of the assembly used. In
474 particular, N50 seemed to better predict mapping percentage compared to average contig length.
475 Turbot shows the longest average contig length, but ranked second in terms of positive mapping
476 matches, in agreement with N50 ranking (Table 2). J. Catchen et al. (2013) showed that in
477 threespined stickleback *de novo* approach yielded a higher number of tags (42,300) than the
478 reference based one (37,600), mostly due to loss of loci that could not be mapped against the
479 reference genome (>4,700). Likewise, in our analysis, using the genome as a reference returned
480 a lower number of tags compared to the *de novo* approach (Table 3). In any case, the number
481 of *de novo*-based tags that mapped correctly to the reference genome was in good agreement
482 with the number of tags identified by the reference based analysis. The larger number of *de*
483 *novo* ddRAD tags might then be explained in part by the incomplete mapping of reads against
484 the reference genome as in the case of threespined stickleback. This seems reasonable
485 considering that the reference sequences used represent only a portion of the entire genomes of
486 the species. Indeed, compared to the genome lengths estimated from the c-values, from 70%
487 (turbot) to 85%(sea bream) of the entire genomes is represented in the references. So, at least
488 part of the *de novo* loci found could be real fragments of the genomes, coming from regions
489 that have been more difficult to sequence and assemble so far. A second possibility is that a
490 fraction of tags, which STACKS identified as separate “loci” in the *de novo* analysis, is likely
491 represented by divergent alleles of the same locus. However, STACKS controls for such
492 phenomenon through the $-M$ parameter and, in the present study, a less conservative value ($-$
493 $M=5$) than the default one ($-M=2$) was set for all species. More likely, *de novo* approach might

494 include some “spurious” loci at individual level. In support of this hypothesis, a filter that
495 exclude loci shared by less than 80% of individuals, filtered out most of *de novo* loci. The origin
496 of these tags is difficult to find but some sources can be the presence of exogenous DNA, e.g.
497 from viral/ bacterial contaminants or from other species. In fact, blasting the tags that did not
498 present matches with sea bream genome showed that around 20% of these tags could come
499 from virus, bacteria, human or from other species analyzed in the laboratory (data not shown).
500 Using a filter that prunes poorly shared loci would exclude these RAD. In any case, when using
501 a *de novo* approach, a further filter based on alignment of tags with potential contaminant
502 species should be implemented as it require little bioinformatic effort and reduces a potential
503 source of background noise in the results. In addition, we cannot exclude the presence of
504 sequencing errors introduced with amplification in library preparation and sequencing steps.
505 While we cannot exclude that these sequences can provide useful information or could be used
506 as dominant markers (Fu et al, 2013), we recognize that they need to be studied more in detail
507 to understand their origin and whether they can have bad effects on certain downstream
508 applications (i.e. those requiring the use of markers shared by a percentage of individuals).
509 Without deeper knowledge of the origin of these sequences, it is therefore advisable to use the
510 above mentioned filters to reduce source of bias in the final filtered datasets. In general, even if
511 in the form of a draft, a reference genome should allow more efficient SNP detection.

512 (iv) *Genotyping precision and error rates*

513 Genotyping reproducibility across technical replicates is one of the most important test to
514 evaluate genotyping methods. A first analysis on over 100 replicates over the three species
515 datasets, showed that “coverage” represented a significant explanatory variable for differences
516 in mismatch rates. In fact, sea bass’ technical replicates, which were characterized by a

517 significantly lower coverage, also showed lower precision than the two other species. The effect
518 of reduced coverage also appears to be affecting samples characterized by a high DNA quality.
519 Davey et al. (2011) suggested at least 30x average coverage depth for reference genome-based
520 analysis and at least 60x coverage depth for *de novo* analysis in order to obtain a complete
521 coverage of all restriction sites in a genome. In addition, Fountain (2016), based on Mendelian
522 inheritance incompatibilities, showed that genotyping errors decreased with increasing
523 coverage from 5x to 30x in both reference based and *de novo* datasets. In the present study, the
524 average coverage for all the three species was higher than that suggested in the studies
525 mentioned, but also the variability across loci was high (36x-386x in sea bass, 31x-2840x in
526 sea bream and 69x-2731x in turbot), which might influence the outcome in term of mismatch
527 rates. However, we couldn't find any significant correlation between mismatch rate and average
528 locus coverage when analyzing results within single datasets (i.e. "species+strategy").
529 The same analysis showed that the SNPs in the reference-based tags are more consistently
530 genotyped than *de novo* ones in both turbot and sea bream. The positive effect of using a
531 reference genome on genotyping reproducibility is an additional one to the advantage of
532 avoiding inflation of tag number described above. More reproducible genotypes are also
533 obtained when *a posteriori* genotype correction was implemented. Both approaches (reference-
534 based analysis and *a posteriori* correction) come at a price as the total number of tags/SNPs
535 analyzed gets reduced, so its use should be considered to obtain more reliable data according to
536 the aims of a particular project. Other issues deserve care when using genetic markers. One of
537 the most important is allelic dropout, that can lead to errors in population statistics, due to biased
538 heterozygosity estimates (Gautier et al., 2013). Some approaches have been proposed to detect
539 loci affected by allelic dropout, such as analysis of coverage, based on the expectation that

540 mutations within the restriction site would result in fewer reads being generated for one allele,
541 thus creating a bi-modal read depth distribution across loci (Cooke et al., 2016). Nevertheless,
542 a preliminary analysis of coverage distribution of our data didn't show the bi-modal distribution
543 expected with the high coverage obtained. This may be due to the fact that coverage also varies
544 with tag length, swamping any signal from allelic drop-out.

545 *Impact of genotyping accuracy on population assignment*

546 The average level of discrepancy between the test datasets used for the comparison was slightly
547 higher than the genotyping error recorded with the different ddRAD bioinformatic approaches,
548 but allowed us to detect the putative threshold at which genotyping imprecision starts affecting
549 downstream analysis, since we recorded both samples with and without assignment or allele
550 frequency deviations. In any case, levels of mismatch between replicates higher than those
551 found in this study (and approaching the threshold identified as causing deviation in
552 downstream analysis) are commonly found when using RAD genotyping (e.g. Forsström et al.
553 2017; Pecoraro et al. 2016). According to our results, the effect of sub-4 % genotyping
554 differences on allele frequency is not significant, while if variation increased (e.g. 4.8 % in sea
555 bream), the resulting allele frequencies were significantly different. Such findings are clearly
556 dependent on factors such as sample size and distribution of variation over loci, but do provide
557 an indication of the point at which genotype variation impacts allele frequency. Population
558 assignment was only affected when levels of genotyping variation were higher; the only
559 discrepancy being recorded for turbot where, despite displaying the lowest overall genotype
560 variation of the three species, one sample with three allele differences (8.3% of 36 alleles), was
561 assigned to different source populations. Again, such results will depend on which loci
562 displayed genotype variation and cannot be used in isolation to define threshold errors.

563 However, the finding does indicate the potential for realistic levels of genotype error to result
564 in significant changes to diagnostic results if not accounted for when evaluating the accuracy
565 of downstream applications.

566 **Conclusions**

567 Application of new genotyping techniques is rapidly increasing as they potentially allow more
568 accurate, easier and less expensive population genetic analysis of any species. However, several
569 issues might affect the quality of the results. In the present study, it was demonstrated that some
570 factors, i.e. DNA fragmentation and archived-fresh samples, affect the throughput in terms of
571 percentage and absolute number of high quality sequence reads in ddRAD datasets. Similarly,
572 actual fragment length and coverage can differ from expectations, leading to redundant loci and
573 loci with too low coverage. Although RAD has been proven to be applicable on non-model
574 species, the use of a preliminary draft genome sequence increase genotyping performance
575 enabling to obtain higher numbers of loci shared between multiplexed individuals. We highlight
576 the critical importance of introducing replicate individuals among samples to assess the
577 performance of the approach used and we demonstrate how variation in genotype datasets can
578 potentially impact the results of downstream population genetic applications. Our results are
579 useful for setting up genotyping project and for considering the features that can affect
580 genotyping throughput and precision.

581 **Data deposition**

582 Raw sequencing data are available at NCBI, with accession numbers SAMN7145243-7145512.

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