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1 Research Article

2 Species composition in the Molobicus hybrid tilapia strain

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22

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24 markers; aquaculture

25

26 # Research Highlights

27 - Novel SNP markers revealed species contribution within a hybrid tilapia
28 line (Molobicus) undergoing selection for growth performance in brackish
29 water.
30 - The backcross base population, developed from feral *Oreochromis*
31 *mossambicus* and GIFT, matched the predicted 3:1 ratio for
32 *O. mossambicus* and *O. niloticus* respectively.
33 - The selected lines showed a significant increase in *O. niloticus*-specific
34 alleles.

35

Abstract

The "Molobicus" hybrid breeding programme was initiated to improve tilapia growth performance in brackish water. The base population was created by backcrossing F_1 *Oreochromis niloticus* GIFT strains × feral *O. mossambicus* to *O. mossambicus* and selective breeding conducted for performance in brackish water with two lines selected in extensive or intensive rearing conditions. A panel of ten diagnostic SNP markers was applied to estimate the species composition at different stages in the Molobicus programme including parental stocks, F_1 and seventh generation fish from the selective lines. The *O. aureus*-specific markers tested revealed zero or negligible contribution from *O. aureus* to all the groups analysed. Feral *O. mossambicus* possessed an estimated 0.98 frequency of *O. mossambicus*-specific alleles, while GIFT samples had an estimated mean frequency of 0.88 *O. niloticus*-specific alleles. Hybrid F_1 GIFT × feral *O. mossambicus* samples demonstrated close to 50:50 allele frequencies from *O. niloticus* and *O. mossambicus* for seven of the eight SNP loci tested. Analysis of the combined seventh generation Molobicus samples revealed a significant excess of *O. niloticus* alleles in six out of the eight SNPs tested, with this trend being more pronounced in the line selected in intensive culture conditions and showing increased body weight. PCR-based SNP assays such as these can be used to inform on the individual species contribution of fish stocks and provide tools for the genetic management of the tilapia species and future breeding programmes.

59 # 1. Introduction

60 Aquaculture production of tilapias (family Cichlidae), native to Africa and the
61 Middle East, is currently the second highest of any finfish group globally after
62 carps (FAO, 2019). Three species belonging to the *Oreochromis* genus
63 predominate: *O. niloticus* (Nile tilapia), *O. mossambicus* (Mozambique tilapia),
64 and *O. aureus* (Blue tilapia). In the second half of the last century, these fish were
65 widely distributed in Asia and other tropical and semi-tropical regions to increase
66 food production due to commercially desirable traits such as high growth rates,
67 ability to survive in different aquatic environments and resistance to disease
68 (Modadugu and Acosta, 2004).

69 Following the introduction of tilapia species outside their native ranges, concern
70 was raised regarding the genetic management and conservation of the farmed
71 broodstock due to introgression (Macaranas et al., 1986) and low effective
72 population sizes (Pullin and Capili, 1988), resulting in poor performance (Eknath
73 et al., 1991). There was therefore a drive to initiate selective breeding
74 programmes (Gjedrem et al., 2012). The Genetic Improvement of Farmed Tilapia
75 (GIFT) project, started in 1988 by WorldFish (then ICLARM) and partners, was
76 the first major breeding programme designed to improve the performance and
77 supply of high-quality *O. niloticus* stocks suitable for both small-scale and
78 commercial aquaculture systems in Asia (Eknath et al., 1998).

79 To ensure a broad genetic diversity, the base *O. niloticus* populations for GIFT
80 were sampled from wild stocks from Africa and farmed tilapia stocks in Asia and
81 Israel. However, the exact species makeup of the founder populations used in
82 GIFT and other tilapia breeding programmes is unknown, due to the likelihood of
83 introgression into the farmed stocks used (Angienda et al., 2011; Firmat et al.,
84 2013; Neira, 2010).

85 Hybridisation between tilapia species has in some cases been intentional to
86 promote desirable traits in aquaculture, as seen in the production of F_1 hybrids
87 using *O. niloticus* (favoured for rapid growth) and *O. aureus* (tolerant to colder
88 temperatures), also popular due to the high male percentage, that now contribute
89 significantly to the total tilapia production in China under variable climatic

90 conditions (Cai et al., 2004). A breeding programme based on hybrid tilapia,
91 called "SaltUNO" or Molobicus, began in the Philippines in 1999 to improve
92 performance in brackish water. The base population was developed from seventh
93 generation GIFT *O. niloticus* strains (selected for growth) and feral
94 *O. mossambicus* (saline tolerant) captured from wild stocks in the Philippines
95 (Mateo et al., 2004 ; Figure 1). The resulting F_1 hybrid (GIFT *O. niloticus* × feral
96 *O. mossambicus* parent) was backcrossed with feral *O. mossambicus* to improve
97 the salinity tolerance, and generations of selective breeding followed from this
98 backcross, targeting increased growth performance (body weight at five months)
99 and passive selection by rearing fish in brackish water (de Verdal et al., 2014).
100 Two selected lines were developed, one selected in extensive culture conditions
101 (fertilised earthen ponds, without additional feed and at low stocking density) and
102 one in more intensive culture (tank based with *ad libitum* feed and at high
103 stocking density).

104 Molecular differentiation of tilapia species is possible using protein-based
105 allozyme loci, but this method requires destructive sampling and the number of
106 informative markers is limited (Sodsuk and McAndrew, 1991). DNA-based
107 markers provide a greater discrimination potential to measure genetic diversity.
108 Methods such as random amplified polymorphic DNA (RAPD; Bardakci and
109 Skibinski, 1994; Dinesh et al., 1996), microsatellite markers (Costa-Pierce, 2003)
110 and PCR based restriction fragment length polymorphism (RFLP; Toniato et al.,
111 2010) have been used to characterise tilapia species, but none of these techniques
112 give sufficient informative markers to confirm the species purity of individual
113 fish or to assess levels of introgression. Mitochondrial DNA (mtDNA) sequencing
114 has also be applied to separate tilapia species (D'Amato et al., 2007; Wu and
115 Yang, 2012) but this is of limited use for studies of hybridisation and
116 introgression as mtDNA is maternally inherited.

117 The advent of high throughput sequencing has allowed the identification of
118 thousands of single nucleotide polymorphism (SNP) markers (Kumar et al., 2012)
119 to assess genetic diversity within populations, differentiate between species and
120 subspecies and map loci associated traits (Palaiokostas et al., 2013; Van Bers et
121 al., 2012; Xia et al., 2014). One such sequencing technique, restriction-site

122 associated DNA sequencing (RADseq), offers a reduced representation of the
123 genome and is able to generate SNP markers randomly distributed throughout the
124 genome adjacent to restriction enzyme cut sites (Davey and Blaxter, 2010). A
125 recent study using a double digest variant of RADseq (Peterson et al., 2012) ,
126 identified species-specific SNPs for ten different tilapiine species and validated
127 24 putative SNP markers for four species of tilapia commonly used in aquaculture
128 using a PCR based SNP assay (Syaifudin et al., 2019).

129 The aim of the present study was to apply selected species-specific SNP markers
130 for three tilapia species (*O. niloticus*, *O. mossambicus* and *O. aureus*) and to
131 assess the species composition at various stages in the Molobicus breeding
132 programme comprising parental feral *O. mossambicus* and GIFT strains, the F_1
133 hybrid and the seventh generation Molobicus hybrid fish selected in two farming
134 systems. These results from the SNP markers provide insights on how the
135 selective pressures present within the Molobicus breeding program shaped the
136 species contribution and genomic profile of the selected hybrid lines.

137

138 # 2. Materials and Methods

139 **2.1 Ethical Statement**

140 Archived fin samples were obtained from the Molobicus and GIFT breeding
141 programmes and approved for analysis at the University of Stirling by the
142 University Animal Welfare and Ethical Review Body (AWERB).

143 **2.2 Sample collection**

144 Fin clip samples stored in 95% ethanol were obtained from fish involved the
145 Molobicus breeding programme in the Philippines (de Verdal et al., 2014). These
146 samples comprised parental stocks of feral *O. mossambicus* (n=23), F_1 hybrid
147 samples (n=20) from the initial crossing between feral *O. mossambicus* and
148 seventh generation GIFT strains, and 58 individuals (derived from 17 families)
149 from the seventh generation (G7) of the Molobicus breeding programme,
150 following selective breeding for increased body weight at five months (derived
151 from the F_1 hybrid backcrossed with *O. mossambicus*). Within the selective
152 breeding programme, there were two separate selected lines, reared in two
153 different farm environments, either an extensive pond-based system (n=24
154 samples) or an intensive tank facility (n=34). An overview of the Molobicus
155 breeding programme design is provided in Figure 1. As no GIFT samples from the
156 parental seventh generation were available for analysis, GIFT broodstock (n=50)
157 from the nineteenth generation (WorldFish Center, Malaysia) were substituted as
158 the closest available material to the parental GIFT stock (Supplementary Table
159 S1).

160 The HotSHOT method was used to prepare crude genomic DNA of the GIFT fin
161 clip samples (Truett et al., 2000). Purified DNA was extracted by a modified salt
162 precipitation method (Syaifudin et al., 2019). Small pieces of fin tissue were
163 digested in 300 µL SSTNE lysis solution (0.3 M NaCl, 50 mM Tris base, 0.2 mM
164 EDTA pH 8.0, 0.2 mM EGTA, 0.5 mM spermidine, 0.25 mM spermine and 0.1%
165 SDS) containing 1.5 µL proteinase K (10 mg/mL) at 55 °C overnight. Lysed
166 samples were treated with 5 µL RNaseA (2 mg/mL) at 37 °C for 1 h and the
167 supernatant centrifuged twice at 21,000 ×*g* after precipitation with 180 µL 5 M
168 NaCl on ice. The resulting DNA was precipitated in an equal volume of
169 isopropanol, washed twice in 70% ethanol and dissolved in TE buffer (10 mM

170 Tris, 1 mM EDTA pH 8.0) until DNA quantification. The quantity and quality of
171 DNA were assessed by measurement on a Nanodrop spectrophotometer (Labtech
172 International Ltd, UK) and by agarose gel electrophoresis. Standardised dilutions
173 of 8 ng/μL DNA were prepared in 5 mM Tris buffer pH 8.0.

174 **2.3 Species-specific diagnostic SNP markers**

175 Ten SNP markers were selected for this study (Syaifudin et al., 2019) based on
176 the ability to clearly distinguish among three species (four with an allele specific
177 for *O. niloticus*, four for *O. mossambicus* and two for *O. aureus*) as indicated by a
178 high frequency of the diagnostic allele (97% for one of the *O. niloticus* markers,
179 100% for the other nine) in the target species and absence of this allele in the
180 other two species based on a test panel of 75 individuals from the three species.
181 Details of the PCR primers are provided in Supplementary Table S2.

182 **2.4 PCR-based SNP genotyping**

183 Individuals were genotyped using KASP (Kompetitive Allele Specific end-point
184 PCR) technology by LGC Genomics Ltd (UK) as detailed previously (Syaifudin
185 et al., 2019). KASP primers were designed, manufactured and supplied at a
186 proprietary concentration by LGC. Either 1 μL HotSHOT preparation or 8 ng of
187 purified DNA template for each assay was dried in a single well of a 96 well
188 white PCR plate (Starlab, UK). The PCR was conducted in a 5 μL total volume
189 with 0.07 μL allele-specific primers in the propriety KASP Master Mix. PCR
190 cycling conditions (TAdvanced thermocycler, Biometra) included an initial
191 denaturation step at 94 °C for 15 min, 10 cycles at 94 °C for 20 s and touchdown
192 65 °C to 57 °C (dropping 0.8 °C each cycle) for 1 min followed by 35 cycles of
193 amplification at 94 °C for 20 s and 57 °C for 1 min. Fluorescence signals were
194 measured at 22 °C using a Quantica® Real Time PCR Thermal Cycler (Techne)
195 and genotypes assigned by allelic discrimination analysis using the Quansoft
196 software v1.121.

197 **2.5 Statistical analysis**

198 Deviation of allele frequency (Chi-square goodness of fit test; Power and Sokal,
199 2011) in the G7 hybrid samples from the expected 1:3 ratio (*O. niloticus*:
200 *O. mossambicus*) in the backcross base population was calculated using an online
201 tool [http://www.socscistatistics.com/tests/goodnessoffit/Default2.aspx]. Principal

202 Component Analysis (PCA) and Discriminant Analysis of Principal Components
203 (DAPC) was carried out on these SNP data using R v3.3.2 (R Core Team, 2019)
204 and an associated R/*adegenet* package v1.4-1 (Jombart, 2008) to model the total
205 variation within the dataset and identify clusters of genetically related individuals
206 within the Molobicus breeding programme.

207

208 # 3. Results

209 The species-specific SNP assays allowed the species contribution of the samples
210 from the Molobicus breeding programme to be assessed. Individual SNP
211 genotypes of the GIFT strains and Molobicus samples for each of the ten markers
212 tested by KASP are listed in Supplementary Table S3. A summary of the genotype
213 distribution and allele frequency of the GIFT, parent and Molobicus strains is
214 presented in Table 1. Among the total fish genotyped for two *O. aureus*–specific
215 markers (n=151), only one copy of an *O. aureus* – specific allele was detected (a
216 single heterozygote for *Oau*966 in the GIFT population). It was therefore
217 concluded that *O. aureus* contribution to the Molobicus samples tested was
218 negligible, signifying the alternate allele for *O. niloticus*-specific markers
219 indicated an *O. mossambicus* allele and vice versa (*i.e.* the alternate allele for
220 *O. mossambicus*–specific markers indicated an *O. niloticus* allele).

221 **3.1 GIFT tilapia**

222 The 50 GIFT tilapia samples (nineteenth generation) were found to possess
223 predominantly the diagnostic allele at the *O. niloticus*-specific SNPs and the
224 alternate allele at the *O. mossambicus*-specific SNPs (combined mean of 0.88
225 *O. niloticus* allele frequency and 0.12 *O. mossambicus* allele frequency, based on
226 the previous assumption that only two species contributed).

227 **3.2 Feral *O. mossambicus* tilapia (parent of Molobicus hybrid)**

228 The majority of the feral *O. mossambicus* (n=22) used as parents for the
229 Molobicus hybrid programme were noted to be homozygous for the diagnostic
230 allele at all four of the *O. mossambicus*-specific SNP markers, and homozygous
231 for the alternate allele at all four *O. niloticus*-specific markers (combined mean of
232 0.98 *O. mossambicus* alleles, 0.02 *O. niloticus* alleles). The one exception, sample
233 MoMo-14-1, presented with a heterozygous genotype for three out of the four
234 *O. mossambicus* SNP markers selected. The corresponding allele frequency for
235 *O. niloticus* specific markers was low (mean 0.03), with the diagnostic marker
236 only evident in two individuals as a heterozygous genotype.

237 **3.3 F1 parental cross (GIFT × feral *O. mossambicus*)**

238 Aside from *Oni*3057 (five homozygotes present for the alternate allele) and

239 *Omo*2007 (one homozygote for the diagnostic allele), all of the F_1 fish were found
240 to be heterozygous for every marker tested (excluding the *O. aureus*-specific
241 markers). Overall, the mean diagnostic allele frequency for the eight diagnostic
242 *O. niloticus* and *O. mossambicus* markers was 0.47 and 0.51 respectively, close to
243 the expected 1:1 ratio (P=0.55 and P=0.84, respectively).

244 **3.4 Seventh generation (G7) Molobicus hybrid tilapia**

245 Given the genotyping results of the parental fish, the backcross base population
246 was predicted to have a 1:3 (*O. niloticus*:*O. mossambicus*) allelic ratio for the
247 eight SNP markers that distinguished between these two species, with the possible
248 exception of *Oni*3057 (due to the 38:63 ratio, of the respective diagnostic and
249 alternate allele in the F_1 samples). Analysis of the genotyping results for the
250 seventh generation (G7) Molobicus hybrid samples in this study (n=58, both lines
251 combined) indicated a significant (P<0.01) deviation from this 1:3 ratio,
252 favouring *O. niloticus*-associated alleles at the expense of the *O. mossambicus*-
253 associated alleles, for six out of the eight SNPs, while the two remaining markers
254 (*Oni*3057 and *Omo*2007) did not deviate from this ratio (data not shown). When
255 the two lines were analysed separately, three of the eight loci showed a significant
256 excess (P<0.01) of *O. niloticus*-associated alleles in the line selected in the
257 extensive pond culture system (n=24), while in the intensive farming system
258 population (n=34), a significant excess (P<0.01) of *O. niloticus*-associated alleles
259 was noted in five out of the eight studied loci and in two further loci at a lower
260 significance level (P<0.05; Table 1).

261 **3.5 Discriminant Analysis of Principal Components (DAPC)**

262 Discriminant Analysis of Principal Components (DAPC) was conducted using
263 R/*adegenet* based on the ten SNP markers. The dataset included the 151 samples
264 from the present study and the genotypes of 60 individuals representing the three
265 pure tilapia species of *O. niloticus*, *O. mossambicus* and *O. aureus* (Syaifudin et
266 al., 2019) as the reference populations. DAPC analysis was able to clearly
267 separate the pure species of *O. niloticus* (coloured dark orange), *O. mossambicus*
268 (dark blue) and *O. aureus* (green) into three distinct groups using both component
269 comparisons (Figure 2). From the current study, the GIFT samples genotyped
270 (light orange) were positioned as a broad cluster closer to *O. niloticus* than to

271 *O. mossambicus*. The feral *O. mossambicus* (light blue), although with an
272 elliptical distribution, overlapped the reference *O. mossambicus* strains. In
273 contrast, the F_1 Molobicus hybrid strains (coloured pink) were placed at an
274 intermediate distance between the pure *O. niloticus* and *O. mossambicus*
275 populations. Following selection, the G7 Molobicus hybrid strains formed two
276 overlapping clusters extending beyond the F_1 hybrids. The discriminant analysis
277 supported the closer association of the G7 Molobicus hybrid strains farmed in the
278 extensive culture system (light grey) relative to the feral *O. mossambicus* parental
279 strains compared to the group reared in the intensive culture system (dark grey)
280 that were positioned more adjacent to the clusters containing the *O. niloticus* pure
281 species and GIFT population.

282

283 # 4. Discussion

284 **4.1 SNP methodology**

285 The identification of tilapia species is of importance for the management of
286 farmed and wild stocks due to the existence of multiple tilapia species and
287 potential hybrids. A panel of ten validated species-diagnostic SNP assays
288 exploiting KASP technology were applied to confirm the genotype of individuals
289 from the Molobicus breeding programme. The number of SNPs applied was a
290 compromise based on the expected species contribution, information gained and
291 minimising the cost within a large-scale breeding programme.

292 KASP technology was chosen for the SNP assays due to the flexibility, low cost
293 and ease of use compared to array-based platforms (Semagn et al., 2014). The
294 HotShot DNA extraction method was able to generate crude DNA template
295 suitable for the PCR assay and offer equivalent performance (data not shown) and
296 a rapid alternative to the longer salt precipitation protocol yielding purified DNA.
297 The accuracy of KASP genotype call was confirmed in the original validation
298 study by the high level of agreement 99.4% found between the PCR based assay
299 and ddRADseq data for the panel of 24 species specific SNPs and 34 tilapia
300 samples (Syaifudin et al., 2019), with disagreement noted by the inherent bias
301 towards homozygotes in the RADseq method (Davey et al., 2013). It is therefore
302 recommended that SNP genotypes derived from KASP assays rather than RADseq
303 studies be relied upon for small scale SNP profiling due to the improved accuracy
304 especially, as in this study, when heterozygotes are anticipated.

305 **4.2 Interpretation of genotype and species contribution**

306 The expectation was that the species contribution involved in the Molobicus
307 hybrid would be primarily from *O. mossambicus* and *O. niloticus*, but two
308 *O. aureus*-specific markers were included as *O. aureus* is another tilapia species
309 that has been widely transferred through aquaculture. Apart from a single copy of
310 the *O. aureus* diagnostic allele in a single GIFT individual, the data did not show
311 any evidence of *O. aureus* contribution to the Molobicus hybrid, so it was
312 assumed that the alternate allele for the *O. niloticus*-specific markers indicated an
313 allele of *O. mossambicus* origin, and vice-versa.

314 **4.3 GIFT broodstock genotype**

315 Based on the selected SNP panel, the KASP results suggested that individuals
316 from the GIFT broodstock population (nineteenth generation) were mainly
317 composed of the *O. niloticus* species (mean 0.82 diagnostic allele frequency) with
318 a minor contribution from *O. mossambicus* (mean 0.07) and negligible
319 contribution from *O. aureus*. These results are in agreement with previous SNP
320 genotyping studies that noted a close association between GIFT and *O. niloticus*
321 individuals (Van Bers et al., 2012; Xia et al., 2015), also reinforced by the close
322 placement of the GIFT population to the reference *O. niloticus* samples following
323 DAPC analysis within this study.

324 On the basis of the SNP markers analysed in the present study, and the
325 assumption (explained above) that only *O. niloticus* and *O. mossambicus*
326 contributed to GIFT, it appears that the nuclear genome of GIFT is around 88%
327 *O. niloticus* and 12% *O. mossambicus*, but with only eight markers and the
328 frequencies per locus ranging from 63 to 100% *O. niloticus*-specific alleles, plus
329 the evidence for selection affecting most of these markers in Molobicus, this is
330 only an estimate. Evidence of introgression by *O. mossambicus* within the GIFT
331 strain has been documented before and the most likely source of *O. mossambicus*
332 introgression would have arisen from the Asian farmed stocks used in the GIFT
333 base population (Taniguchi et al., 1985). This minor level of introgression by
334 *O. mossambicus* is consistent with previous genotyping studies suggestive of a
335 lower than 20% *O. mossambicus* admixture in certain GIFT individuals when
336 assignment testing was applied to estimate the genetic structure of GIFT samples
337 based on combined mtDNA and microsatellite data (McKinna et al., 2010),
338 microsatellite data (Sukmanomon et al., 2012) and SNP sequencing (Xia et al.,
339 2014), but not when mtDNA haplotypes were considered alone (40% O.
340 mossambicus mtDNA; McKinna et al., 2010).

341 McKinna et al. (2010) also concluded that 2 of 30 GIFT tilapia analysed (7%) had
342 *O. aureus* mitochondrial DNA haplotypes, but it seems likely that these originated
343 from West African *O. niloticus*, which have mtDNA haplotypes typical of
344 *O. aureus* despite having nuclear genomes related to *O. niloticus* (Rognon and
345 Guyomard, 2003; Syaifudin et al., 2019). This current study supports a lower

346 (negligible) contribution of *O. aureus* within GIFT tilapia. Previous genotyping
347 studies have also noted GIFT individuals that contained trace levels of genetic
348 variation suggestive of *O. aureus* or a third species involvement other than
349 *O. niloticus* and *O. mossambicus* in alignment with the present findings
350 (Sukmanomon et al., 2012; Xia et al., 2014). Further discriminatory genotyping
351 studies will be required to assess the level of possible introgression by *O. aureus*
352 within the GIFT population.

353 **4.4 Feral *O. mossambicus* genotype**

354 It is perhaps surprising that the species-specific SNP profiles implied that the
355 feral *O. mossambicus* sourced from the Philippines and used as parents for the
356 Molobicus breeding programme had only a trace contribution from *O. niloticus*
357 (0.03 mean allele frequency). This was largely due to two individuals that were
358 multiple heterozygotes. Although *O. mossambicus* was the first tilapia species
359 introduced into the country in the mid-1950s, the findings suggest that this
360 particular population of feral *O. mossambicus* has been able to maintain a high
361 level of genetic purity in the wild despite the later introduction of domesticated
362 and inevitable release of feral *O. niloticus* into the same environment (Pullin et
363 al., 1997).

364 **4.5 F1 Molobicus hybrid stock genotype**

365 The GIFT samples analysed were derived from a later generation (nineteenth)
366 than used in the development of Molobicus, which may account for why the F_1
367 fish (seventh generation GIFT × feral *O. mossambicus*) were found to be
368 heterozygous for the markers tested, with one exception. SNP *Oni*3057, where
369 0.38 *O. niloticus* diagnostic alleles were observed in the F_1 individuals, was one
370 of the two loci with the lowest frequency of *O. niloticus* diagnostic alleles in the
371 GIFT samples. The almost uniform observation of heterozygotes for the other
372 seven loci in the F_1 strains led to testing the seventh generation Molobicus data
373 against an expected 1:3 (*O. niloticus*:*O. mossambicus*) allelic ratio.

374 **4.6 G7 Molobicus species contribution and culture system**

375 De Verdal et al. (2014) showed that the Molobicus line selected for performance
376 in a tank-based culture system at high stocking density, with an average salinity
377 of 2.2 ppt, and fed *ad libitum* responded to a greater extent to selection for body

378 weight than the line reared in the earthen ponds at low stocking density with no
379 external feed input and a lower salinity level of 1.5 ppt. This correlated with a
380 greater shift towards *O. niloticus*-specific alleles in the intensive line that
381 responded more strongly to selection (mean *O. niloticus* allele frequency 0.44 in
382 the intensively reared line vs 0.33 in the extensively reared line, with seven
383 significant increases in *O. niloticus* allele frequency ($P<0.05$) from the predicted
384 1:3 *O. niloticus*: *O. mossambicus* ratio compared to three respectively). Analysis
385 of the SNP dataset by DAPC also supported these findings, shown by the relative
386 position of the G7 population reared under intensive conditions adjacent to the
387 *O. niloticus* species compared to the extensive G7 population cluster positioned
388 closer to the parental feral *O. mossambicus* group.

389 4.7 Diagnostic SNP markers and trait association

390 It could be anticipated that the *O. niloticus* genome would carry more alleles for
391 faster growth at genes affecting this trait, while *O. mossambicus* could possess
392 more allelic variants for greater salinity tolerance at genes affecting this trait,
393 given the known attributes of these species. Similarly, the species associated
394 SNPs used in this study and distributed throughout the genome could reflect these
395 and other traits particular to a species, as seen by the two basic patterns of
396 species-specific allele frequency (1:3 or 1:1) according to the species-specific
397 marker tested. Certainly, the average body weight of the Molobicus hybrids
398 farmed in the intensive system at G4 was reported to be increased and growth
399 more rapid than the low input environment (de Verdal et al., 2014). Enhanced
400 growth is a known attribute of the *O. niloticus* species and the main selection
401 drive within the GIFT selection programme (Ponzoni et al., 2011), so perhaps a
402 proportion of the *O. niloticus* SNP panel could reflect a growth advantage. Efforts
403 on ongoing to unravel the genetic basis for growth selection with polymorphisms
404 in the growth hormone gene implicated in *O. niloticus* (Jaser et al., 2017) and
405 multiple linkage groups (LGs) associated with growth in saline tolerant hybrid
406 tilapia derived from *O. mossambicus* and Asian red tilapia (Lin et al., 2016).

407 Likewise, a subset of the SNP markers could have been influenced by the
408 differing levels of salinity between the two culture systems, although both hybrid
409 populations were able to tolerate the brackish conditions, outside the optimum

410 range (0 to 1.0 ppt) for *O. niloticus* (Villegas, 1990). However, questions could be
411 raised as to whether the salinity was actually at a sufficient level in the Molobicus
412 breeding programme to impose strong differential selection favouring regions of
413 the *O. mossambicus* genome associated with salinity tolerance. Given the benefits
414 of extending the culture of tilapia into brackish environments, different
415 approaches have been put forward to maximise this resource in coastal regions
416 and where water sources are limited (Cnaani and Hulata, 2011). Salt tolerance has
417 been assessed using GIFT strains grown in seawater (Ridha, 2008) and using
418 hybrids between *O. niloticus* and *O. mossambicus* in Thailand (Kamal and Mair,
419 2005). Recent studies have attempted to characterise the underlying genetic
420 mechanisms involved in tilapia salinity tolerance and identified Prolactin I (PRL
421 I; Streelman and Kocher, 2002; Velan et al., 2015) and the Enhancer of Polycomb
422 Homolog 1 (EPC1) as possible candidate genes involved in osmoregulation (Gu
423 et al., 2018; Su et al., 2020).

424 The advent of high throughput sequencing and completion of the genome
425 assembly for commercially important tilapia species such as *O. niloticus* (Conte
426 et al., 2017) should help accelerate the identification and genetic manipulation of
427 key traits. SNP datasets are available for the three species included here and Red
428 tilapia (Kajungiro et al., 2019; Van Bers et al., 2012; Xia et al., 2014). This
429 technology was demonstrated in a later whole genome sequencing study able to
430 locate the signatures of selection in multiple LGs most common in non-coding
431 regions as well as known growth-related pathways in genetically improved tilapia
432 lines (Xia et al., 2015). The ten diagnostic SNP markers employed in the current
433 study also represent multiple LGs throughout the genome and are positioned in
434 non-coding regions, however their functional significance is unknown. Further
435 analysis of whether there have been differential changes across the Molobicus
436 genome, in terms of the contribution of *O. mossambicus* and *O. niloticus*, would
437 require a more detailed analysis using large SNP sets or resequencing, and to
438 associate genomic regions with the traits under selection.

439 **Conclusions**

440 A set of ten species-specific SNP markers diagnostic for the commercially
441 important tilapia species *O. niloticus*, *O. mossambicus* and *O. aureus* was applied

442 to hybrids involved in the Molobicus breeding programme, developed from GIFT
443 × *O. mossambicus* crosses and selected for growth performance in brackish water.
444 Following seven generations of selection, the SNP profiling results indicated that
445 there had been a shift in the original species contribution within the hybrid
446 population in favour of *O. niloticus* at the expense of *O. mossambicus* alleles and
447 that this effect was more pronounced in the line selected in an intensive culture
448 system, which also showed a greater response to growth selection, compared to
449 the line selected in an extensive farming environment. This is the first case study
450 to demonstrate the utility of species-specific SNP markers in the identification of
451 tilapia species and assessment of changes within tilapia hybrids under selection.
452 Equally, this discriminatory SNPs method offers a particular value in assessing
453 the threat of hybridisation in native populations of tilapias (FAO, 2019).

454

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464

465 ## Author contributions

466 The study was designed by HdV and DJP. WR, NM, JAHB, BJM, MS, HdV and
467 JBT contributed samples. The laboratory work was conducted by KLB. KLB, KT,
468 MB and SW conducted the data analysis. KLB, KT and DJP drafted the initial
469 manuscript. All authors read, edited and approved the manuscript.

470

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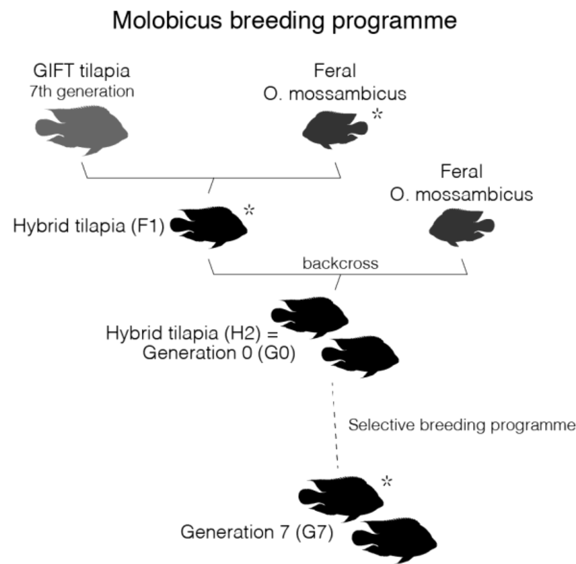
662

663 # Figures

664 Figure 1 (1 column, 85 mm x 85 mm)



665

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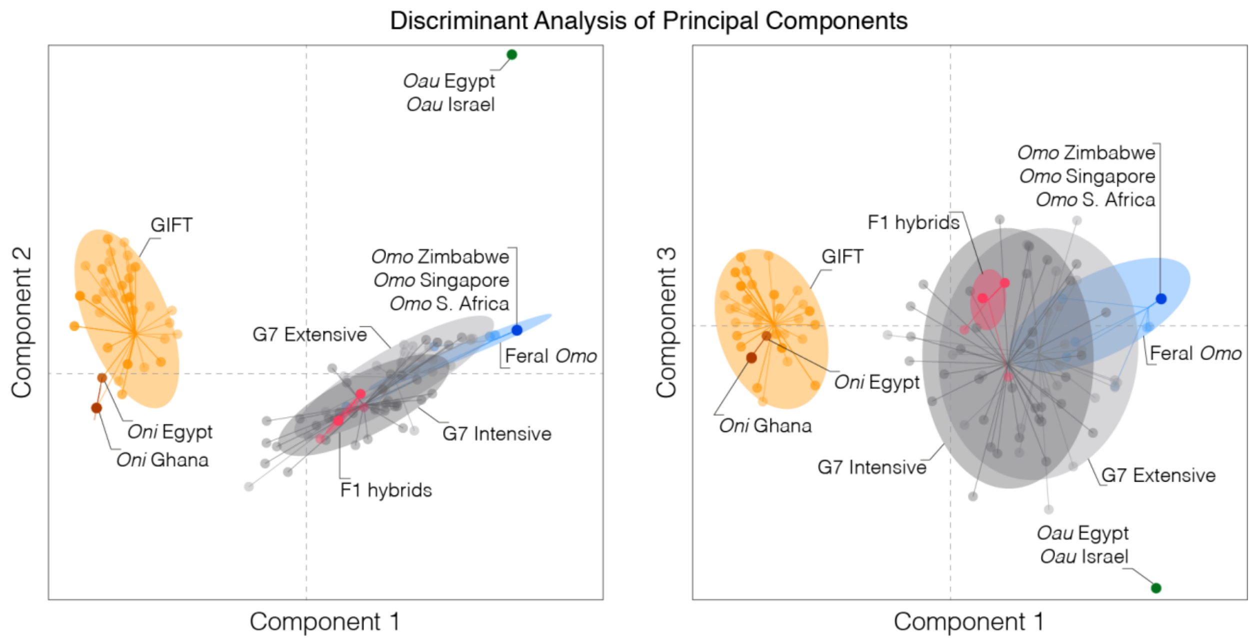
667 Figure 2 (2 columns, 80 mm x 167 mm)



668

669

670 # Figure Legends

671 **Figure 1. Overview of the Molobicus breeding programme**. An F_1 hybrid was
672 initially produced from parental GIFT strains (seventh generation) and feral
673 *O. mossambicus*, and backcrossed with *O. mossambicus*. Hybrid families
674 underwent seven generations of selective breeding based on increased harvest
675 weight in either extensive or intensive culture conditions. Asterisks (*) represent
676 the three sampling points for this study.

677 **Figure 2. Scatterplot of a Discriminant Analysis of Principal Component**
678 **(DAPC)**, using two principal components to separate GIFT and Molobicus strains
679 based on ten species diagnostic SNP markers of the three species *O. niloticus*
680 (*Oni*), *O. mossambicus* (*Omo*) and *O. aureus* (*Oau*). Left panel: Component 1 and
681 Component 2; Right panel: Component 1 and Component 3. Strains of pure
682 tilapia species from different populations acted as a reference and were colour
683 coded (*Oni*, dark orange, origin Egypt and Ghana*; Omo*, blue, origin Zimbabwe,
684 Singapore and South Africa; *Oau*, green, origin Egypt and Israel), GIFT strains
685 (light orange), feral *Omo* Molobicus parental strains (light blue), Molobicus F_1
686 hybrid (pink) and G7 Molobicus hybrid strains (light grey, extensive culture; dark
687 grey, intensive culture).

688

Tables

Table 1. Genotype and allele frequency of Molobicus samples. Ten putative species-diagnostic SNP markers (*O. niloticus* n=4, *Oni*; *O. mossambicus* n=4, *Omo*; and *O. aureus* n=2, *Oau*) of GIFT, *O. mossambicus* parent and hybrid Molobicus strains are shown. For each marker, the number of observed genotypes (Hom, homozygous diagnostic, alternate or heterozygous) and the allele frequency (diagnostic and alternate) is reported. * *P*<0.05; ** *P*<0.01 (1:3 *Oni*:*Omo* expected ratio).

	Oni3057	*Oni5782*	*Oni9497*	*Oni2675*	*Omo2007*	*Omo2657*	*Omo3481*	*Omo7956*	*Oau966*	*Oau9418*
GIFT (n=50)										
Hom. diagnostic	27	50	40	20	4	0	0	0	0	0
Heterozygous	22	0	10	23	15	4	0	0	1	0
Hom. alternate	1	0	0	7	31	46	50	50	49	50
Diagnostic frequency	0.76	1.00	0.90	0.63	0.20	0.04	0.00	0.00	0.01	0.00
Alternate	0.24	0.00	0.10	0.37	0.80	0.96	1.00	1.00	0.99	1.00
O. mossambicus parents (n=23)										
Hom. diagnostic	0	0	0	0	23	22	22	22	0	0
Heterozygous	1	2	1	1	0	1	1	1	0	0
Hom. alternate	22	21	22	22	0	0	0	0	23	23
Diagnostic frequency	0.02	0.04	0.02	0.02	1.00	0.98	0.98	0.98	0.00	0.00
Alternate	0.98	0.96	0.98	0.98	0.00	0.02	0.02	0.02	1.00	1.00
F1 Molobicus hybrids (n=20)										
Hom. diagnostic	0	0	0	0	1	0	0	0	0	0
Heterozygous	15	20	20	20	19	20	20	20	0	0
Hom. alternate	5	0	0	0	0	0	0	0	20	20
Diagnostic frequency	0.38	0.50	0.50	0.50	0.53	0.50	0.50	0.50	0.00	0.00
Alternate	0.63	0.50	0.50	0.50	0.48	0.50	0.50	0.50	1.00	1.00
Molobicus G7 [extensive culture] (n=24)										
Hom. diagnostic	2	7	3	2	14	16	7	10	0	0
Heterozygous	6	13	10	6	8	6	11	8	0	0
Hom. alternate	16	4	11	16	4	2	6	6	24	24
Diagnostic frequency	0.21	0.56	0.33	0.21	0.75	0.79	0.52	0.58	0.00	0.00
Alternate	0.79	0.44**	0.67	0.79	0.25	0.21	0.48**	0.42**	1.00	1.00
Molobicus G7 [intensive culture] (n=34)										
Hom. diagnostic	1	13	4	7	24	8	8	13	0	0
Heterozygous	23	12	18	19	6	19	13	11	0	0
Hom. alternate	10	9	12	8	4	7	13	10	34	34
Diagnostic frequency	0.37	0.56	0.38	0.49	0.79	0.51	0.43	0.54	0.00	0.00
Alternate	0.63*	0.44**	0.62*	0.51**	0.21	0.49**	0.57**	0.46**	1.00	1.00

698 # Supporting Information

699 **Supplementary Table S1. Details of sample origin**. For each sample, sample
700 reference, species identification, country of collection and strain origin are
701 provided.

702 **Supplementary Table S2. SNP markers**. Details of the PCR primers, diagnostic
703 alleles, frequency and associated dyes for the ten SNP assays (from Syaifudin et
704 al., 2019).

705 **Supplementary Table S3. SNP markers genotypes**. Lists the genotypes of each
706 sample for the ten SNP assays.

707