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Parentage assignment in the critically endangered European sturgeon (*Acipenser sturio*) based on a novel microsatellite multiplex assay: a valuable resource for restocking, monitoring and conservation programs

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HAL Authorization

1 **Parentage assignment in the critically endangered European**
2 **sturgeon (*Acipenser sturio*) based on a novel microsatellite**
3 **multiplex assay: a valuable resource for restocking, monitoring and**
4 **conservation programs**

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27 **SUMMARY:** The only remaining population of the critically endangered European sturgeon,
28 *Acipenser sturio*, is located in the Gironde basin (France). A restoration program initiated 20
29 years ago has allowed more than one and a half million individuals to be stocked. Effective
30 monitoring of this population is a key prerequisite in ensuring the sustainability of this
31 species in the wild. We report the development of a novel microsatellite multiplex assay for
32 genetic monitoring of *A. sturio*. Diversity of a set of 18 loci was low to moderate, with a
33 number of alleles and observed heterozygosity ranging from 4 to 7 and 0.33 to 0.74
34 respectively, depending on markers. A set of captive-born progeny of known relatives (n=72)
35 was used to examine the efficiency of this assay in assigning parentage to offspring. Three
36 different programs were used. Correct assignment success was generally high (above 90%),
37 but differed between programs. Parentage analysis of individuals captured in the Gironde
38 estuary (n=193) demonstrated that most offspring (91.2%) are unambiguously allocated to
39 parent pairs from the broodstock. Our research provides an efficient and accurate method
40 for the genetic monitoring of the restocking program, but also for others aspects of
41 conservation, including genetic diversity evaluation, effective population size estimation,
42 and inbreeding assessment.

43

44 INTRODUCTION

45 Sturgeons are among the most threatened groups of fish in the world, with most species at
46 risk of extinction (Rochard et al. 1990, Birstein 1993 and IUCN 2015). Because of some life
47 characteristics (late age at maturity, diadromous life cycle for some species), the
48 conservation of many sturgeon species has relied on long-term breeding and restocking
49 programs – generally over several decades (Carmona et al. 2011, Williot 2011). The genetic
50 monitoring of such species, also characterized by a large number of progeny, therefore
51 depends upon time and cost-effective protocols for routine screening. There is currently a
52 captive breeding stock of European sturgeon (*Acipenser sturio*) located at the Irstea
53 experimental station in Saint-Seurin-sur-l'Isle, France. This stock, first created in 1994, is
54 made up of wild-born adults and juveniles. Its aim is to secure the survival of the species and
55 provide a basis for subsequent releases into the wild (Williot et al. 2007).

56 The first individuals from this stock were released back into the wild in 1995 (Williot et al.
57 2002) with further releases taking place 12 years later. Since then, more than one and a half
58 million larvae and juveniles have been released into the Gironde basin. One priority of the
59 restoration program is to characterize the estuarine portion of the population and estimate
60 the efficiency of restocking by monitoring the population in the Gironde estuary (Rochard et
61 al. 2001, Lochet et al. 2004, Acolas et al. 2011). Because the probability of *A. sturio*
62 reproduction in the wild has been low since 1994, an almost closed system is expected, in
63 which most of the captured individuals would belong to the captive-born stock. Since 2007,
64 restocking plans have been devised whereby the progeny of unique parent pairs that are
65 released into the wild at different stages (i.e. larvae, juvenile) and in different locations
66 (Gironde, Dordogne) can be identified. This plan also allows the survival of these different
67 offspring to be assessed based on these criteria. The development of genetic tagging is
68 necessary to provide unambiguous identification of supplemental fish, and to evaluate
69 survival and/or reproductive success in *A. sturio*.

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75 Of the many different methods used for fish tagging, genetic monitoring would appear to be
76 the most efficient for young stages. This is because it is not constrained by issues of size, as is
77 the case with transponders and external devices (Feldheim et al. 2002, DeHaan et al. 2007).
78 It also has an advantage over mass chemical markers, the effectiveness of which are also
79 reduced when applied to very young sturgeons (Lochet et al. 2011).

80 Genetic tagging using microsatellites consists of creating a genetic profile for each captured
81 individual, allowing them to be assigned to a given population or parental pair (Lukacs &
82 Burnham 2005). This approach is popular in the management of conservation programs
83 involving aquatic organisms (Hansen et al. 2001, Liu & Cordes 2004, Moghim et al. 2013,
84 Abdul-Muneer 2014). For example, it is often used to analyze the proportion of wild-born
85 individuals and captive-born individuals in the natural environment (Poteaux et al. 1999,
86 Bravington & Ward 2004, Meraner et al. 2014). Microsatellite markers are also powerful
87 tools in captive management (O'Reilly & Kozfkay 2014) because they enable the genetic
88 characterization of captive broodstock (Koljonen et al. 2002, Machado-Schiaffino et al. 2007,
89 Cooper et al. 2009), as well as guiding mating strategies based on relatedness (Kozfkay et al.
90 2007, Nielsen et al. 2007, O'Reilly & Kozfkay 2014), inbreeding levels (Borrell et al. 2007),
91 and breeding success (e.g., McLean et al. 2007, Hoskin et al. 2015, Sard et al. 2015).

92 The number of markers needed to obtain reliable results will depend upon the genetic
93 variability of the species studied and the taxonomic level or geographic scale of the analysis
94 required (Frankham et al. 2002, Wan et al. 2004). Restocking and conservation programs
95 generally focus on species characterized by population declines and low genetic diversity.
96 Relatively large numbers of microsatellites are often necessary to gain sufficient reliability
97 and statistical power. Multiplex assays (i.e. the amplification of several microsatellite loci in
98 single PCRs) are therefore relevant for the development of standardized screening protocols
99 for cost-effective genetic analyses (Neff et al. 2000, Renshaw et al. 2006, Morvezen et al.
100 2013, Panagiotopoulou et al. 2014). Numerous microsatellites have already been developed
101 and used to study wild and captive populations of sturgeon species, for conservation or in
102 commercial breeding programs (May et al. 1997, McQuown et al. 2000, King et al. 2001,

103 Henderson-Arzapalo & King 2002, Zane et al. 2002, Welsh et al. 2003, Zhu et al. 2005, Börk
104 et al. 2007, Forlani et al. 2007, Fopp-Bayat & Woznicki 2008, Waldman et al. 2008, Dudu et
105 al. 2011, Moghim et al. 2012, Wozney et al. 2012, Moghim et al. 2013, Zeng et al. 2013,
106 Georgescu et al. 2014, Liu et al. 2014, Panagiotopoulou et al. 2014, Wirgin et al. 2015).

107

108 For *A. sturio*, there are currently no available specific markers and no standardized multiplex
109 protocols. Only a handful of preliminary studies have described species diversity based on
110 non-specific (Ludwig et al. 2004, Ludwig 2005, Williot et al. 2007, Chassaing 2010, Berrebi &
111 Cherbonnel 2011, Chassaing et al. 2011) or unpublished (Tiedemann et al. 2011) markers.
112 One of the characteristics of the last remaining population of this species is its low genetic
113 diversity (Chassaing 2010), which makes it difficult to design reliable protocols with sufficient
114 resolution power.

115 In this paper, we present a method based on three multiplexes totaling 18 polymorphic
116 microsatellite loci for its use in the conservation and restocking programs of *A. sturio*.
117 Specifically, the aims of the present study were to (1) Develop and validate an optimized
118 multiplex protocol as a standard genotyping tool for individual genetic tagging (2) Test our
119 assay on 193 individuals captured in the Gironde Estuary (South West of France) in order to
120 assess the feasibility of our methodology in real monitoring condition, and (3) Discuss the
121 usefulness of our assay for the genetic monitoring of both the captive breeding and
122 restocking programs for the long term management of the species.

123

124 **MATERIALS AND METHODS**

125 **Biological material and DNA extraction**

126 We sampled a total of 109 *A. sturio* from the captive broodstock (BROODSTOCK) (N= 19
127 males and 18 females) composed of 33 wild adults and juveniles captured during the 1970-
128 1994 period and 4 adults from the first cohort born in captivity in 1995, and 72 captive
129 progeny (FARM) issued from 30 known parents for the validation of parentage testing.
130 Additionally, 193 individuals, obtained by trawling in the Gironde estuary (Aquitaine, France)
131 during population monitoring campaigns (2009-2014) were sampled (CAPTURED) (Acolas et

132 al. 2011). These captured individuals (juveniles and adults of length between 40-130 cm;
133 mean=70 cm) may come either from captive cohorts of the restocking program that involve
134 a total of 27 known mating pairs between 2007 and 2014, or from reproduction events in
135 the wild. For all individuals, a piece of fin was individually collected and preserved in 95%
136 ethanol.

137 **Selection and test of sturgeon microsatellites in *A. sturio***

138 DNA extraction was performed using the Chelex extraction method (Walsh et al. 1991).

139 Because there were no available microsatellite markers specifically developed for *A. sturio*, a
140 total of 118 microsatellite sequences developed for other sturgeon species were screened in
141 the European sturgeon (see Appendix 1). Firstly, simplex PCR amplifications were tested on
142 24 individuals in a 25 μ L reaction volume composed of 0.5 units of Promega Taq polymerase,
143 1.5 mM MgCl₂, 0.2 mM of each deoxyribonucleotide, 1 X buffer and 12.5 pmol of each
144 primer. PCR was performed for all loci with the following thermocycling regime: 95°C for 5
145 min, then (95°C 40 s, 54°C 40 s and 72°C 40 s) \times 34 cycles, and 72°C for 10 min. Amplification
146 success was checked by running PCR products on an agarose gel. Loci which successfully
147 amplified were then tested with the same PCR conditions. Forward primers were labelled
148 with a fluorescent dye (6-FAM™, Cy3, NED™ or TET) in simplex or duplex reactions. The PCR
149 products were separated on a denaturing 6% polyacrylamide gel and then scanned on a
150 fluorescence image analyzer, FMBIO II Multi-View (Hitachi Software, Tokyo, Japan). A total of
151 34 loci (see Supplementary material S1) were selected and tested in Multiplex reactions on
152 24 individuals. PCR amplifications were carried out with the Type-it Microsatellite PCR Kit
153 (Qiagen™) in a 10.5 μ L reaction volume containing 6.25 μ L of Type-it Multiplex PCR Master
154 Mix, 1.25 μ L primer mix (each primer at 2 μ M) with forward primers labelled at 5' end using
155 fluorescent dyes (6-FAM™, HEX™ and NED™), 3 μ L RNase-free water and 4 μ L Chelex DNA
156 extraction. Thermal cycling profile followed manufacturer conditions (Qiagen™) and PCR
157 products were separated on a capillary sequencer (either ABI 3130 XL or ABI 3500 XL,
158 Applied Biosystem™). Fragment lengths were assessed with Peak Scanner v1.0 and
159 GeneMapper v4.0 softwares (Life Technologies™). Selection of the best quality markers was
160 done first, discarding those with more than two alleles per individual or with very low
161 variability. To reduce the probability of genotyping errors, we prioritized those with high

162 reliability, clear patterns of the expected range, and high signal intensity. The remaining 18
163 loci were amplified in three multiplex (MLPX1, MLPX2 and MLPX3; see Table 1).

164

165 **Statistical analysis and optimization of a microsatellite multiplex assay**

166 Basic genetic parameters including allele frequencies, numbers of alleles per locus (A) and
167 observed (H_o) and expected (H_e) heterozygosity were calculated for BROODSTOCK
168 individuals using CERVUS v.3.0 (Kalinowski et al. 2007). Linkage disequilibrium between pairs
169 of loci was tested with GENEPOP 4.0 (Rousset 2008) using the Markov chain method (10 000
170 dememorization steps, 100 batches, 5000 iterations) and Fisher's exact test; a sequential
171 Bonferroni correction for multiple testing was applied (Rice 1989). Null allele frequency
172 estimates were computed using CERVUS 3.0. The success of amplification was calculated as
173 the percentage of positive PCR for each locus over the whole sample. The 18 selected loci
174 were classified in increasing order of probability of identity (PID) (Waits et al. 2001) which is
175 a good predictor of the true probability of correct parentage assignment. PID values were
176 calculated using the GIMLET software (Valière 2002) under "sibs" and "unrelated"
177 relatedness scenarios. The polymorphic information Content (PIC) for each locus, as well as
178 the combined non-exclusion probabilities over loci, for first parent (NE-1P), second one (NE-
179 2P), parent pair (NE-PP), unrelated individuals (NE-unrel) or siblings (NE-sibs) were
180 calculated using CERVUS v.3.0 (Kalinowski et al. 2007).

181 **Parentage testing and applicability for the restocking program**

182 To validate the best parentage assignment protocol, our microsatellite multiplex assay was
183 first tested on 72 progeny of known parents (FARM) with three commonly used programs
184 that perform different algorithms: CERVUS v3.0 (Marshall et al. 1998, Kalinowski et al. 2007);
185 PASOS v1.0 (Duchesne et al. 2005) and PAPA 2.0 (Duchesne et al. 2002). CERVUS and PASOS
186 allow for incomplete parental sampling (i.e. open systems) while PAPA is better designed for
187 parentage assignment in closed systems (i.e. all parents are sampled). To select the
188 minimum number of loci necessary for reliable assignment, all three programs were tested
189 using several sets of an increasing number of markers, classified in the order of PID values.

190 CERVUS uses a likelihood-based approach to assign parental origin combined with simulation
191 of parentage analysis to determine the confidence of parentage assignments. Likelihood

192 score ratios (LOD or Delta) estimate the likelihood that the candidate parent is the true
193 parent divided by the likelihood that the candidate parent is not the true parent. Before
194 proceeding to the parentage assignment, simulations were run in CERVUS to determine the
195 distribution of the critical values of Delta or LOD score for 80% and 95% confidence levels.
196 The following simulation parameters for 100,000 offspring were chosen for our studied
197 organism: numbers of candidate parents (BROODSTOCK) incremented by a 10-15%
198 corresponding to putative non-sampled parents (i.e. 20 candidate mothers and 22 candidate
199 fathers), 10% of candidate parents with a relatedness coefficient of 0.1, 98% of candidate
200 parents sampled, 99% loci typed with a 1% error rate. Confidence levels obtained from
201 simulations were used for true paternity screening of the 72 progeny (FARM). In PASOS and
202 PAPA, likelihoods are calculated for each potential parental pair and the allocation of a
203 descendant is based on the search for the most likely pair among all potential pairs of known
204 parents. For the analysis with those two programs, we allowed one locus genotype
205 mismatch between parents and offspring (i.e. offset=1) as suggested by the author (P.
206 Duchesne, Personal Communication). Assignments of FARM samples were compared to the
207 true parent's pairs, which was defined as true assignment (i.e. TRUE). The difference
208 between both assignments is considered as the error rate associated with the method.
209 Assignment success, true assignment, and error rates were also compared among the three
210 programs.

211 To test the feasibility of our assay in tracking the parental origins of cohorts released into the
212 wild, parentage analysis was conducted on 193 individuals captured in the Gironde Estuary
213 (CAPTURED).

214

215 **RESULTS**

216 **Microsatellite variability**

217 Of the 118 primer pairs tested, 80 (68%) gave an amplification product, and among those, 33
218 (41%) were polymorphic (i.e. two or more alleles per locus) (*Appendix 1*). To decrease the
219 putative error rate associated with allele scoring, we decided to exclude 15 loci based on
220 unclears signal on the capillary sequencer (AfuG113, AoxB34, AoxD241, AciG198, Afu19, and

221 AoxD297), or low reliability (AfuG184, Ag16). Other seven markers were omitted because of
222 their reduced variability (Afu54, Ag20, Aox23, Aox27, AoxD54, AS002, Spl101). Statistics for
223 the 18 selected loci are given in Table 1. No overlapping of allele size ranges was observed
224 within any of the three multiplexes and the amplification success was high for all loci (Table
225 1). No significant linkage disequilibrium was detected between pairs of markers after
226 Bonferroni corrections. Overall polymorphism was moderate to low, with the number of
227 alleles (N_a) varying from two (Ag39) to seven (Ag10) alleles per locus, H_e from 0.46 to 0.90
228 and H_o from 0.33 to 0.74. Null allele frequency was displayed for a single marker, but at a
229 very low rate (AoxD188; ≤ 0.05). PIC values varied between loci, between 0.37 and 0.77.
230 Although the mean number of alleles per locus was low ($n=4$), the potential of these 18 loci
231 used in combination to distinguish individuals was high, as indicated by the low cumulative
232 probability of identity (PID) over loci of respectively of $5E-12$ or $1E-05$, assuming all
233 individuals are unrelated (PID-unrel) or siblings (PID-sibs). Values of non-exclusion
234 probabilities for unrelated (NE-unrel) and siblings (NE-sibs) were also low, at $1.18E-12$ and
235 $4.62E-06$, respectively, as well as the combined non-exclusion probabilities for the first
236 parent, second parent and parent pairs of $1.6E-02$, $4.9E-05$ and $2.5E-06$, respectively.
237 Amplification success was high for all loci (Table 1).

238 **Validation of parentage assignment in *A. sturio***

239 Simulations in CERVUS result in high assignment rates, with 98% assignments of parent pairs
240 at both strict (95%) and relaxed (80%) levels. Only 2% of simulated offspring remained
241 unassigned. Individual genetic tagging was successful in identifying the parental origins of
242 most individuals originating from the breeding program (FARM). The parentage assignments
243 of these offspring using the three methods (CERVUS, PASOS, and PAPA) are shown in Figure
244 1. Overall, assignment success was high and increased as the number of loci used went up,
245 but for all programs the highest level of assignment success was obtained for 17 loci (Figure
246 1A). For FARM, parentage analysis using CERVUS generated 100% parent pair assignments
247 (Figure 1B). The results of assignment tests in CERVUS showed that most individuals (95.8%)
248 were assigned to the parent pair matching the parent database information (i.e. named
249 TRUE assignment) at high (strict, 95%) or lower (relaxed, 80%) level of confidence, but that a
250 few individuals ($N=3$ out of 72, 4.2%) were wrongly assigned. Using PASOS, 93.1 % of
251 offspring were unambiguously assigned to their true parents ($N=67$ out of 72), while four

252 (5.5%) had a parentage assignment corresponding to “uncollected” mother or father
253 (Duchesne et al. 2005) and one was assigned to wrong parental pair (i.e. 1.4%). PAPA gave
254 65 correct assignments (90.3 %) (N=65 out of 72) and 2 wrong assignments (2.7%), while the
255 assignment of the remaining individuals was ambiguous (i.e. the highest likelihood is shared
256 by several parental pairs, see Duchesne et al. 2002), and is therefore the least powerful
257 program in parentage testing for *A. sturio*. These results indicate that error rates associated
258 with the methodology (i.e. the difference between assignment and TRUE assignment) are
259 low to moderate and range from 9.7% to 4.2%, depending on the program used.

260

261 **Genetic monitoring of the stocked population**

262 The results of analyses of the captured individuals (CAPTURED, N=193) were compared
263 between CERVUS and PASOS, the two most powerful programs. Parentage testing of
264 CAPTURED using CERVUS generated 99 % parent pair assignments. A single juvenile was not
265 assigned with a sufficient level of confidence (<80 % confidence). Using PASOS, assignment
266 success for parent pairs was 96%. Eight individuals were assigned to one unsampled father
267 or mother. When successful assignments were compared to the 23 known mating pairs from
268 the breeding program, 17 individuals (8.8%) and 18 individuals (9.3%) were assigned to non-
269 existing parental pairs, for CERVUS and PASOS, respectively. Among those, 15 individuals
270 were common among both programs. These 15 assignments (7.8 %) might be the result of
271 errors associated with the method (estimated above between 6.9% and 4.2%), as well as a
272 possible wild origin.

273

274 **DISCUSSION**

275 Parentage assignment in endangered species – which are generally genetically impoverished
276 – calls for a difficult balance to be struck between having enough polymorphic markers to
277 provide the necessary resolution power and managing the time and expense associated with
278 prolonged laboratory work (Harrison et al. 2013). In this study, we aimed to test and
279 estimate a minimum number of loci for accurate parental assignment in *A. sturio* and
280 optimize a multiplexing approach for routine screening. Because no specific markers were
281 available for the species, we tested more than a hundred markers developed for other
282 sturgeon species (i.e. "crosspriming"). We found that more than 40% of the markers were

283 polymorphic and that successful crosspriming concerned markers developed for either very
284 close (i.e. *A. oxyrinchus*) or more distant (*A. gueldenstaedtii*) species (Appendix 1).
285 Crosspriming has proved to be effective among sturgeon species (Rajkov et al. 2014) and its
286 use has been largely extended in previous studies (May et al. 1997, King et al. 2001, Moghim
287 et al. 2012).

288 Assignment success with microsatellites also relies on accurate genotyping. The high
289 mutation rate of microsatellites is often associated with scoring errors (Pompanon et al.
290 2005), as well as frequent mismatches of offspring genotypes with those of their parents,
291 which may lead to incorrect assignment and this will even increase while increasing the
292 number of markers used. Generally, the number of typed loci should be chosen as a
293 compromise between the probability of identity (more resolution) and the probability of
294 error (loss of correct assignment). This stresses the need for the technique to be optimized
295 and validated prior to its application to ensure accuracy in parent-offspring assignments
296 (Nielsen et al. 2001). Although our results support the previous documented low genetic
297 diversity for *A. sturio* (Chassaing et al. 2010, 2016), the final optimized assay of three
298 multiplexes allowed efficient and reliable amplification of 18 loci with PID and non-exclusion
299 probabilities sufficiently low to assure proper individual and parental identification. Our
300 multiplex assay was successful in identifying the parents of a set of 72 progeny, whose
301 parental origin was known (Figure 1). The analysis of cumulative loci revealed that 17 loci of
302 the 18 were required in order to meet the maximum assignment rate. This has been
303 observed elsewhere for individual assignment (Roques et al. 1999) and illustrates that
304 adding extra loci may increase the proportion of erroneous genotypes, thus leading to a
305 larger proportion of incorrect allocations. This also pointed out that Aox45 might be a poorly
306 segregating locus. Considering the best assignment obtained with CERVUS, only three
307 individuals were assigned to the wrong parental pairs (i.e. 4.2%), which is a satisfying result
308 given the very low genetic diversity of the species, some level of relatedness among parents
309 and the high probability of scoring errors. This value could be considered the maximum error
310 rate associated with the present genetic assay (i.e. corresponding to laboratory, sample
311 handling, or data transcription's errors) and should be taken into account in future analyses.
312 These results support the potential of these programs to establish parent-offspring

313 relationships even when some genotypes are incomplete, incorrect or missing (Kalinowski et
314 al. 2007).

315 This study further clearly highlights the power of this multiplex assay for the monitoring of
316 the *A. sturio* restocking program. Genetic tagging proved to be a reliable tool for species
317 conservation programs requiring a high number of individuals to be monitored (Andreou et
318 al. 2012). For *A. sturio* population monitoring, tagging systems such as PIT (passive
319 integrated transponder) or WOT tags (external loop tags, wire on tag) are efficient methods,
320 but are limited by the size of fish (Acolas et al. 2011). Genetic tagging clearly avoids the
321 problem of tag loss, and can be used at all stages. Furthermore, genetic monitoring is a
322 widely used tool to estimate successful reintroduction, establishment and reproduction of
323 individuals released into the wild (e.g. Perrier et al. 2013, Sard et al. 2015, Schreier et al.
324 2015). Our results provide compelling evidence that most individuals captured in the Estuary
325 descended from captive breeders (91.2%) and thus give positive signs of reintroduction
326 success of captive-born *A. sturio* into the wild. In addition, according to our previous error
327 estimation (4.2%) a small estimated proportion (i.e. $100 - (91.2 + 4.2) = 4.6$ % corresponding to
328 9 individuals) might be issued from reproduction in the wild which need to be confirmed by
329 further investigations. This indicates that *A. sturio* survival will depend mostly upon
330 successful captive breeding and stocking, and stresses the importance of such genetic assay
331 for the careful and intensive ex-situ management of this species.

332 Our genetic assay further enables the study of other aspects of genetic management such as
333 monitoring levels of genetic variability in the captive stock, estimating inbreeding
334 coefficients or breeding success. For example, the comparison of the genetic composition
335 and levels of inbreeding between the captive stock and the released cohorts are important
336 clues in conservation breeding programs (Wilson et al; 2012, Schreier et al. 2015). The
337 captive breeding stock of *A. sturio* in Europe is shared through a joint conservation program
338 between IRSTEA in Bordeaux and IGB (Institute of Freshwater Ecology and Inland Fisheries)
339 in Berlin, and sturgeons from France are regularly transferred to be released into the Elbe
340 and to complement the German captive stock in Germany (Kirschbaum et al. 2011). This
341 set of multiplex assays has the potential to become a promising tool to standardize genetic
342 information and coordinate scientific tools to assist in genetic management of *A. sturio* in
343 the future.

344

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355 captive stock.

356 **FIGURES**

357 **Figure 1.** Comparison of assignment success (in percentage) of captive-born progeny (FARM)
358 using three programs (PASOS, CERVUS, and PAPA). A) TRUE assignment rates for several sets
359 of increasing number of markers (from 12 to 18) classified in increasing order of probability
360 of identity (PID). TRUE refers to assignment success when compared to known data of
361 parental pairs. B) Assignment, TRUE assignment and error rates for the multiplex assay of 17
362 markers.

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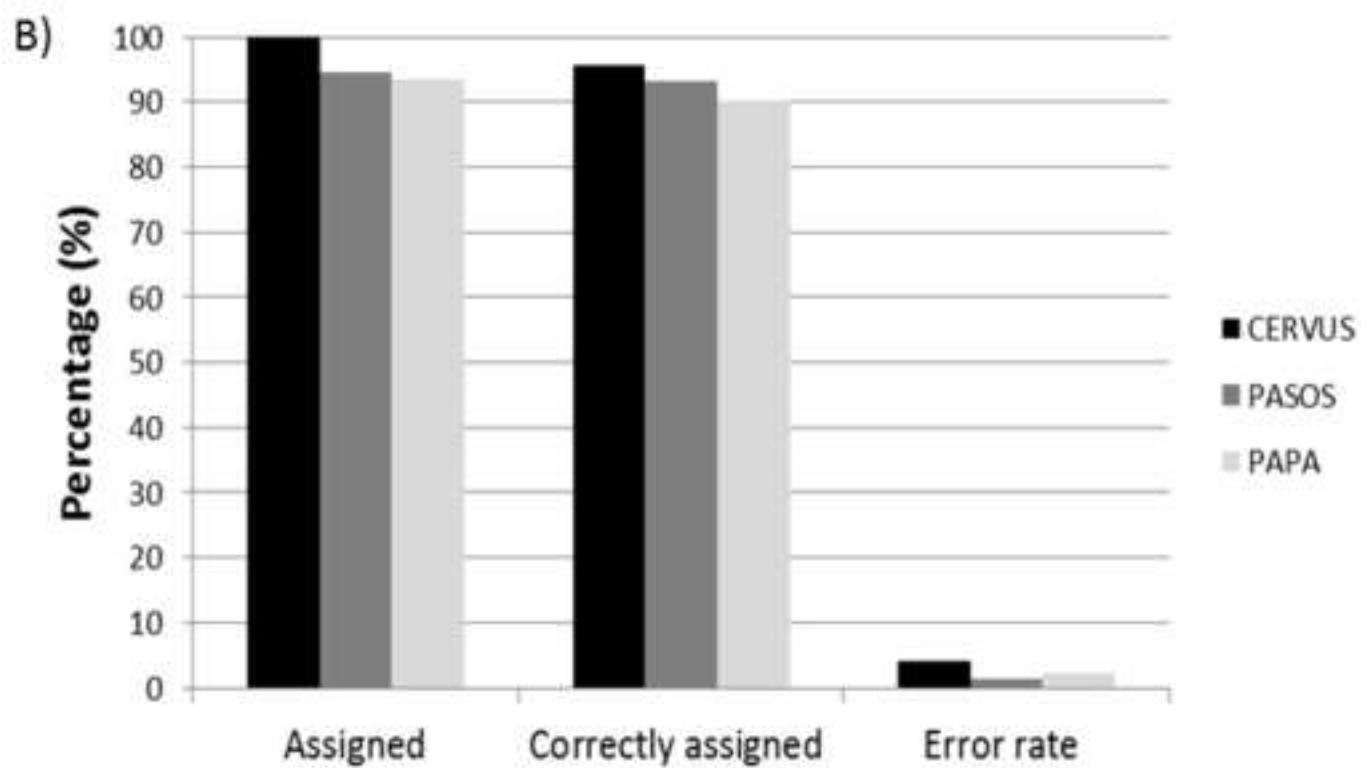
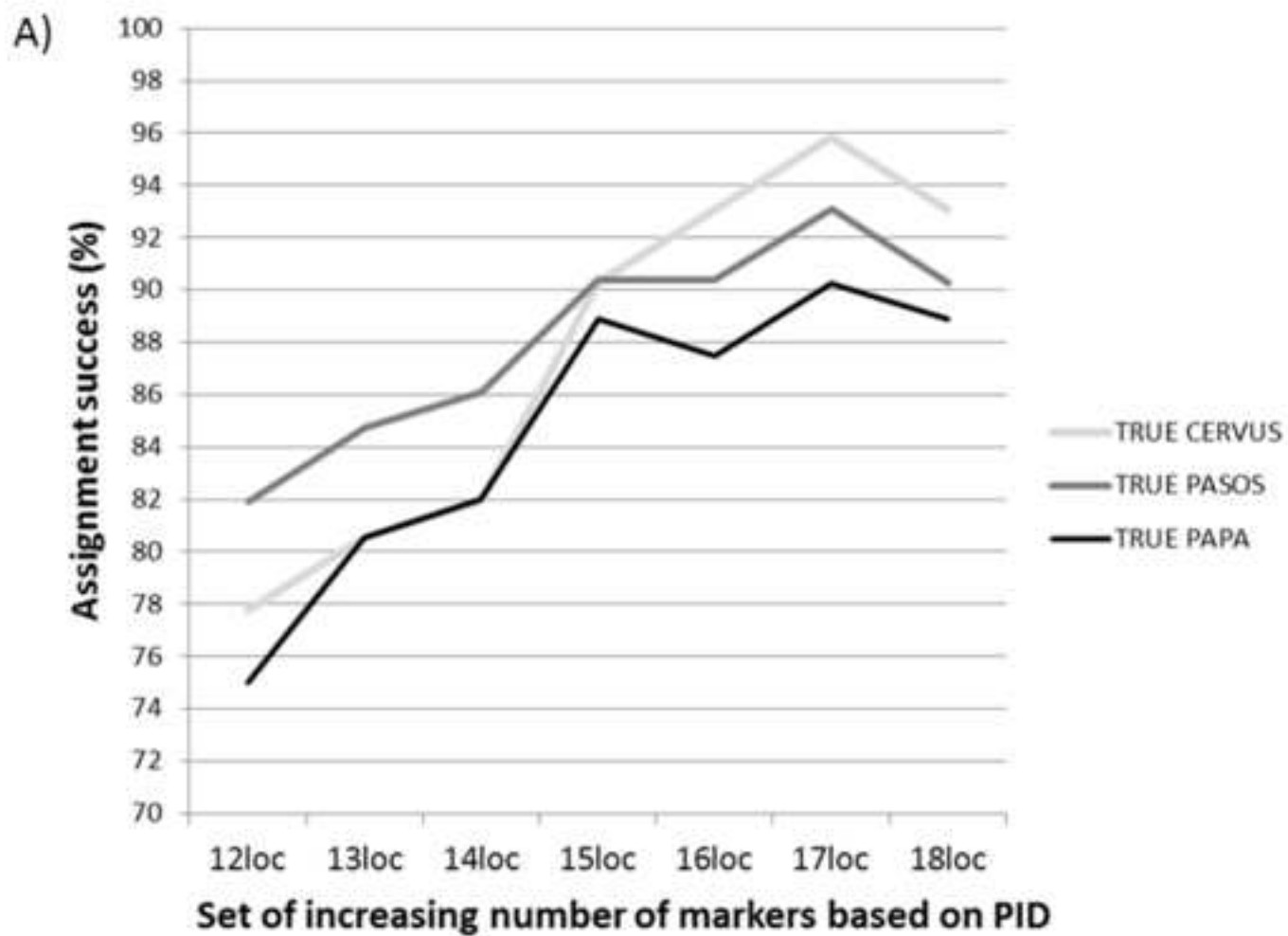


Table 1: 18 microsatellites selected for *Acipenser sturio* multiplex assay and diversity in N=50. Annealing temperature (Ta), Forward (F*, labelled with fluorescent dye) and reverse (R) primer sequences, expected (Hexp) and observed (F null), rates of amplification success (in percentage), expected (Hexp) and observed (F null)

Name	Fluorescent dye	MPLX	Ta (oC)	Primer sequence F* (5'-3')
AOXD64	FAM	MPLX1	55	TTGTCCAATAGTTTCCAACGC
AOXD165	FAM	MPLX1	55	TTTGACAGCTCCTAAGTGATACC
AG10	FAM	MPLX1	55	AACAAGTTCTTACCTCGATTTTGG
AG47	NED	MPLX1	55	GCGAAACGACTCCCTTAACA
AOXD32	NED	MPLX1	55	CAGATTTAAGTAAGATAAGCATCAGC
AOXD44	HEX	MPLX1	55	ACCGAGTTTCAAATCAAATAGC
AOXD234	HEX	MPLX1	55	AACTGGCTTTGTGATTGATCC
AG14	FAM	MPLX2	55	GCTGTCCCATTAGCTGATCC
AOXD188	FAM	MPLX2	55	TGAAGTCATTGGTGATGTGTATG
AOXC55	NED	MPLX2	55	GCAAGGTGTATTAAGTGGACC
AOXD161	HEX	MPLX2	55	GTTTCAAATGATTGAGAAAATGC
AOX45	HEX	MPLX2	55	TTTGTGTAGGGAAAATACCCTTG
AG38	FAM	MPLX3	55	AAACAGGTATAAAAATGTTGCTTGTG
SPL106	FAM	MPLX3	55	CACGTGGATGCGAGAAAATAC
AG28	NED	MPLX3	55	CCATCAGCAGCTTCAACTCA
AG39	HEX	MPLX3	55	CATCTGGGAAGCAAGTGGAG
AFU68	HEX	MPLX3	55	TTATTGCATGGTGTAGCTAAAC
AN20	HEX	MPLX3	55	AATAACAATCATTACATGAGGCT
Overall mean				

0 captive individuals. Microsatellites are amplified in three multiplex (MLPX1, MLPX2, MLPX3);
 10 primer sequences; Number of alleles per locus (A), allelic range in bps (range, bp), null allele frequency
 (Hobs) heterozygosity and probability of identity under unrelated (PID unrel) and sibling (PII)

Primer sequence R (5'–3')	A	Range (bp)	F (null)	Amplification success rate (%)	Hobs
TGTGCTCCTGCTTTTACTGTC	5	103-145	-0.126	99.7	0.58
AAAGCCCTACAACAAATGTCAC	4	174-190	-0.072	100	0.73
GAGATTTGAACAAGACAGGAGGA	7	227-287	-0.075	97.2	0.80
ACAGGTCTTGGGCTTTCCTC	6	110-122	-0.126	100	0.59
AAAGCAGCTTGACATAACGG	4	156-204	-0.098	98.9	0.70
TGAAACTGCTGTGCAATAAGAG	3	144-160	-0.044	100	0.60
TGAAGCAAAGGGTATTATTTGAG	4	197-221	-0.111	97.9	0.60
GACAGAGGATGTTTCTGTGAGC	5	188-204	-0.167	100	0.70
ATGGAAATGTTTTATGGTAATGTG	3	244-252	0.049	99.7	0.57
CGACCCTGTAAAGGAGTAAGC	4	117-141	-0.023	100	0.67
TGAGACAGACACTCTAGTTAAACAGC	5	157-173	-0.114	100	0.72
TGAGTGCAGCCCTACTGCTC	4	198-222	-0.037	98.9	0.42
TCAGAAAGAGTTTAGTACGCATGG	5	190-202	-0.087	99.3	0.59
GGGGAGAAAAGTGGGGTAAA	3	227-235	-0.002	99.7	0.51
ACATGCACGTATGCACGC	5	152-172	-0.105	96.6	0.44
CTCGATGGAACCCAGAAAAG	2	123-125	-0.183	99.7	0.47
AGCCCAACACAGACAATATC	4	142-168	-0.138	99.3	0.48
TGGTCAGTTGTTTTTTATTGAT	5	186-214	-0.168	99.7	0.47
	4			99.2	0.57

ncy
D sibs) scenarios .

Hexp	PID unrel	PID sibs	References
0.60	1.21E-08	3.92E-04	Henderson-Arzapalo and King 2002
0.80	8.74E-03	1.54E-01	Henderson-Arzapalo and King 2002
0.90	7.00E-02	3.69E-01	Rajkov et al. 2014
0.72	9.21E-07	2.91E-03	Rajkov et al. 2014
0.74	2.48E-05	1.25E-02	Henderson-Arzapalo and King 2002
0.66	5.15E-08	7.55E-04	Henderson-Arzapalo and King 2002
0.68	2.17E-07	1.48E-03	Henderson-Arzapalo and King 2002
0.90	1.66E-04	2.84E-02	Rajkov et al. 2014
0.50	8.75E-10	1.10E-04	Henderson-Arzapalo and King 2002
0.70	4.45E-06	5.77E-03	Henderson-Arzapalo and King 2002
0.88	1.14E-03	6.52E-02	Henderson-Arzapalo and King 2002
0.52	1.88E-12	5.53E-06	Henderson-Arzapalo and King 2002
0.72	3.10E-09	2.04E-04	Rajkov et al. 2014
0.54	3.01E-10	6.39E-05	McQuown et al. 2005
0.46	1.29E-11	1.42E-05	Rajkov et al. 2014
0.66	5.06E-12	8.74E-06	Rajkov et al. 2014
0.64	1.06E-10	3.82E-05	Welsch et al. 2003
0.62	3.83E-11	2.32E-05	Zane et al. 2002
0.65	1.88E-12	5.53E-06	

Appendix 1: List of 118 sturgeon loci tested in this study

POLYM: positive amplification and more than one allele per locus, UNCL

SIZE: Size exceeding 500 bps; NO AMP: no amplification

Locus	Amplification	Species
AfuG113	POLYM	<i>Acipenser fulvescens</i>
Ag01	UNCLEAR	<i>Acipenser gueldenstaedtii</i>
Ag12	DUPLI	<i>Acipenser gueldenstaedtii</i>
Ag15	DUPLI	<i>Acipenser gueldenstaedtii</i>
AoxB34	POLYM	<i>Acipenser oxyrinchus</i>
AoxD241	POLYM	<i>Acipenser oxyrinchus</i>
AciG22	UNCLEAR	<i>Acipenser transmontanus/Acipenser mer</i>
AfuG198	MONOM	<i>Acipenser fulvescens</i>
Afug51	MONOM	<i>Acipenser fulvescens</i>
Ag02	MONOM	<i>Acipenser gueldenstaedtii</i>
Ag08	MONOM	<i>Acipenser gueldenstaedtii</i>
Ag09	MONOM	<i>Acipenser gueldenstaedtii</i>
Ag19	MONOM	<i>Acipenser gueldenstaedtii</i>
Ag21	MONOM	<i>Acipenser gueldenstaedtii</i>
Ag22	MONOM	<i>Acipenser gueldenstaedtii</i>
Ag24	MONOM	<i>Acipenser gueldenstaedtii</i>
Ag25	MONOM	<i>Acipenser gueldenstaedtii</i>
Ag26	MONOM	<i>Acipenser gueldenstaedtii</i>
Ag35	MONOM	<i>Acipenser gueldenstaedtii</i>
Ag40	MONOM	<i>Acipenser gueldenstaedtii</i>
Ag45	MONOM	<i>Acipenser gueldenstaedtii</i>
AoxD242	MONOM	<i>Acipenser oxyrinchus</i>
AS004	MONOM	<i>Acipenser sinensis</i>
AS021	MONOM	<i>Acipenser sinensis</i>
AS043	MONOM	<i>Acipenser sinensis</i>
Psp-26	MONOM	<i>Polyodon spathula</i>
Psp-29	MONOM	<i>Polyodon spathula</i>
Spl-163	MONOM	<i>Scaphirhynchus platyrhynchus</i>
AciG 56	NO AMP	<i>Acipenser transmontanus/Acipenser mer</i>
AciG 93	NO AMP	<i>Acipenser transmontanus/Acipenser mer</i>
AciG110	NO AMP	<i>Acipenser transmontanus/Acipenser mer</i>
AciG142	NO AMP	<i>Acipenser transmontanus/Acipenser mer</i>
AciG48	NO AMP	<i>Acipenser transmontanus/Acipenser mer</i>
AciG4	NO AMP	<i>Acipenser transmontanus/Acipenser mer</i>
AciG76	NO AMP	<i>Acipenser transmontanus/Acipenser mer</i>
AfuG115	NO AMP	<i>Acipenser fulvescens</i>
Ag03	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag04	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag06	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag07	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag17	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag18	NO AMP	<i>Acipenser gueldenstaedtii</i>

Ag27	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag29	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag30	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag32	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag33	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag37	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag42	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag43	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag44	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag46	NO AMP	<i>Acipenser gueldenstaedtii</i>
Ag50	NO AMP	<i>Acipenser gueldenstaedtii</i>
An16	NO AMP	<i>Acipenser naccarii</i>
AnacE4	NO AMP	<i>Acipenser naccarii</i>
AoxB28	NO AMP	<i>Acipenser oxyrinchus</i>
AoxD170	NO AMP	<i>Acipenser oxyrinchus</i>
LS34	NO AMP	<i>Acipenser fulvescens</i>
Psp-12	NO AMP	<i>Polyodon spathula</i>
Psp-20	NO AMP	<i>Polyodon spathula</i>
Psp-21	NO AMP	<i>Polyodon spathula</i>
Spl-100	NO AMP	<i>Scaphirhynchus platyrhynchus</i>
Spl-104	NO AMP	<i>Scaphirhynchus platyrhynchus</i>
Spl123	NO AMP	<i>Scaphirhynchus platyrhynchus</i>
Spl-168	NO AMP	<i>Scaphirhynchus platyrhynchus</i>
Spl170a	NO AMP	<i>Scaphirhynchus platyrhynchus</i>
AfuG123	UNCLEAR	<i>Acipenser fulvescens</i>
AfuG72	UNCLEAR	<i>Acipenser fulvescens</i>
Ag05	UNCLEAR	<i>Acipenser gueldenstaedtii</i>
Ag13	UNCLEAR	<i>Acipenser gueldenstaedtii</i>
Ag36	UNCLEAR	<i>Acipenser gueldenstaedtii</i>
Ag41	UNCLEAR	<i>Acipenser gueldenstaedtii</i>
Ag48	UNCLEAR	<i>Acipenser gueldenstaedtii</i>
Ag49	UNCLEAR	<i>Acipenser gueldenstaedtii</i>
AnacC11	UNCLEAR	<i>Acipenser naccarii</i>
AnacD3	UNCLEAR	<i>Acipenser naccarii</i>
Aox12	UNCLEAR	<i>Acipenser oxyrinchus</i>
AoxD161	POLYM	<i>Acipenser oxyrinchus</i>
AoxD242	UNCLEAR	<i>Acipenser oxyrinchus</i>
AciG198	POLYM	<i>Acipenser transmontanus/Acipenser megalopterus</i>
Afu19	POLYM	<i>Acipenser fulvescens</i>
Afu39	UNCLEAR	<i>Acipenser fulvescens</i>
Afu54	POLYM	<i>Acipenser fulvescens</i>
Afu68	POLYM	<i>Acipenser fulvescens</i>
AfuG184	POLYM	<i>Acipenser fulvescens</i>
Afug41	UNCLEAR	<i>Acipenser fulvescens</i>
Ag10	POLYM	<i>Acipenser gueldenstaedtii</i>
Ag14	POLYM	<i>Acipenser gueldenstaedtii</i>
Ag16	POLYM	<i>Acipenser gueldenstaedtii</i>
Ag20	POLYM	<i>Acipenser gueldenstaedtii</i>

Ag28	POLYM	<i>Acipenser gueldenstaedtii</i>
Ag31	UNCLEAR	<i>Acipenser gueldenstaedtii</i>
Ag38	POLYM	<i>Acipenser gueldenstaedtii</i>
Ag39	POLYM	<i>Acipenser gueldenstaedtii</i>
Ag47	POLYM	<i>Acipenser gueldenstaedtii</i>
An20	POLYM	<i>Acipenser naccarii</i>
Aox23	POLYM	<i>Acipenser oxyrinchus</i>
Aox27	POLYM	<i>Acipenser oxyrinchus</i>
Aox45	POLYM	<i>Acipenser oxyrinchus</i>
AoxC55	POLYM	<i>Acipenser oxyrinchus</i>
AoxD165	POLYM	<i>Acipenser oxyrinchus</i>
AoxD172	UNCLEAR	<i>Acipenser oxyrinchus</i>
AoxD188	POLYM	<i>Acipenser oxyrinchus</i>
AoxD242	MONOM	<i>Acipenser oxyrinchus</i>
AoxD234	POLYM	<i>Acipenser oxyrinchus</i>
AoxD297	POLYM	<i>Acipenser oxyrinchus</i>
AoxD32	POLYM	<i>Acipenser oxyrinchus</i>
AoxD44	POLYM	<i>Acipenser oxyrinchus</i>
AoxD54	POLYM	<i>Acipenser oxyrinchus</i>
AoxD64	POLYM	<i>Acipenser oxyrinchus</i>
AS002	POLYM	<i>Acipenser sinensis</i>
Spl101	POLYM	<i>Scaphirhynchus platyrhynchus</i>
Spl-106	POLYM	<i>Scaphirhynchus platyrhynchus</i>
Spl-113	UNCLEAR	<i>Scaphirhynchus platyrhynchus</i>
Ag11	SIZE	<i>Acipenser gueldenstaedtii</i>
Ag23	SIZE	<i>Acipenser gueldenstaedtii</i>
Ag34	SIZE	<i>Acipenser gueldenstaedtii</i>
An77	SIZE	<i>Acipenser naccarii</i>
