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1 **Title:** An infraspecific dimension of bioindication? Comparison between genotypes and
2 ecological distribution of *Potamogeton coloratus*

3

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16

17 **Highlights:**

18 • *Potamogeton coloratus* has a low genetic diversity and no regional structure in France.

19 • It mainly occurs in small streams and pools with low temperatures and NH₄⁺ contents.

20 • It tolerates large variations in nitrate and phosphate concentrations.

21 • Its ecological niche is divided between lotic and lentic habitats.

22 • There is no correlation between these habitat categories and genotypic lineages.

23

24 **Abstract:**

25 The role of within-species genetic variability and ecotypes has been poorly documented in
26 research on water bioindication. Some studies already show that macrophytes could present
27 genetic lineages with different optima for specific water parameters. In the present study, we
28 tested this assumption on *Potamogeton coloratus* Hornem., a macrophyte described as
29 characteristic of oligotrophic waters and threatened by freshwater eutrophication. Species
30 population genetics, water parameters, and macrophyte and macroinvertebrate communities
31 were analysed in 29 sites among three eco-regions of the eastern parts of France: Rhine Plain,
32 Rhône Valley and Provence. The observed diversity of habitats can be divided into two main
33 categories: the lotic hydrosystems such as springs and small watercourses often fed by

34 groundwater, and the lentic waterbodies such as shallow pools or pond sides. Sampling sites
35 were characterised by alkaline (mean pH 7.5 ± 0.4) and cool waters (mean 15.8 ± 4.1 °C) with
36 low N-NH₄⁺ concentration (median value of 20 µg/L), but large variation in other parameters
37 (e.g. nitrates, phosphates). We found two main AFLP genotypic lineages, despite a poor
38 genetic variability mainly distributed between populations, without regional structuring nor
39 within-population diversity. We also sequenced only two ribotypes based on the nrDNA ITS.
40 Genotypes, ribotypes and habitat categories were not correlated, arguing that the divergence
41 of genotypic lineages is not due to ecotypic differentiation for *P. coloratus*. We highlight a
42 putative event of long-distance dispersal, which illustrates the species ability for
43 recolonization. Finally, we provide some suggestions for its conservation facing global
44 changes.

45

46 **Keywords:** oligotrophic species, macrophytes, bioindication, ecotypes, genotypes

47

48 **1. Introduction**

49 Anthropogenic eutrophication threatens biodiversity and stability of freshwater
50 ecosystems, degrading water quality, food resources and other ecosystem services (Mueller et
51 al., 2016; Le Moal et al., 2019). To limit this degradation, several countries (e.g. European
52 Union under the Water Framework Directive; European Commission 2000) have called for
53 the monitoring of surface waters using chemical and ecological indicators, based on a
54 faunistic group, as fishes or invertebrates, and a floristic group, as diatoms or macrophytes
55 (*i.e.* aquatic plants visible to the naked eye). Thus several national standardized methods of
56 bioindication based on macrophytes have been then published, e.g., Germany (Schaumburg et
57 al., 2004), France (Haury et al., 2006), UK (Willby et al., 2009), and Poland (Szozkiewicz et
58 al., 2010). They are usually based on the theoretical relationship between plant community
59 changes along a trophic gradient in hydrosystems (Carbiener et al., 1990).

60 However, this cornerstone assumption for plant bioindication was questioned (Demars
61 et al., 2012), with the alternative one that aquatic plant communities are far more dependent
62 on other physical and chemical river parameters – such as water velocity, river size, alkalinity
63 – than directly on eutrophication (Demars and Edwards, 2009; Steffen et al., 2014). The deep
64 and inextricable correlations between all these parameters along the river continuum call for a
65 meticulous disentangling of underlying processes in river eutrophication and vegetation

66 changes (O’Hare et al., 2018). Some of them must be placed at the infraspecific level: which
67 range of nutrients is tolerated by a given species? This seemingly simple question is actually
68 hardly solvable due to synergistic effects of environmental variables on species response, and
69 the heterogeneous species sensibility depending on nutrient identity.

70 Another emerging question is about ecotypes of a species differing in nutrient
71 preferences (Matthews et al., 2018). An ecotype corresponds to an infraspecific group of
72 individuals adapted to a specific environment. This adaptative process commonly found for
73 terrestrial plants could disrupt bioindication diagnostic based on species preferences as a fixed
74 value. Such infraspecific resolution has already allowed to identify correlation between
75 genetic diversity and habitat types (Han et al., 2014), or links between ecotypes and
76 genotypes. For example, several studies argue for an evolutionary divergence between
77 freshwater and brackish water populations of *Stuckenia pectinata* (L.) Börner, probably due to
78 ecological adaptation rather than geographical isolation (Nies and Reusch, 2005; Triest et al.,
79 2010). Another example was found between genotypes of *Myriophyllum spicatum* L., which
80 showed contrasting sensibilities to copper contamination, correlated with different growth
81 strategies (Roubeau Dumont et al., 2019, 2020). Few studies have linked genotypic distinction
82 within species to varying concentrations of the main nutrients involved in eutrophication, i.e.
83 nitrate, ammonium or phosphate (Bastlová et al., 2004). Besides, these studies often speculate
84 that plant individuals collected from different locations correspond to different genotypes,
85 rather than using molecular markers to robustly distinguish genotypes. Finally, ecotypic
86 adaptations are not necessary detected by neutral markers classically used for genotyping (e.g.
87 SNPs, microsatellites, AFLPs), except if they lead to a genetic isolation of ecotypes.
88 However, a systematic ecological distinction of two sympatric genotypes in the field would
89 represent a supported prediction of ecotypes.

90 In Europe, the fen pondweed, *Potamogeton coloratus* Hornem. (Potamogetonaceae), is
91 often considered as the most oligotrophic-specific macrophyte, as indicated in the French and
92 German bioindication standards (Macrophyte Biological Index for Rivers – IBMR, Haury et
93 al., 2006; Trophic Index of Macrophytes – TIM, Schneider and Melzer, 2003). More
94 specifically, this rhizomatous hydrophyte with floating and submerged leaves is particularly
95 sensitive to ammonium (Glänzer, 1974). This sensitivity to eutrophication might be linked to
96 its threatened status in several countries such as the Czech Republic, France, Germany,
97 Netherlands, Sweden, Switzerland and Turkey. In contrast, the species is more abundant and
98 found in meso-eutrophic conditions in the British Isles. To date, just two genetic studies from

99 the same authors have targeted *P. coloratus* using isozymes (Hollingsworth et al., 1995;
100 Gornall et al., 1998). The first study found a poor genetic variation among 12 British
101 populations, but the second one focusing on a finer scale showed a structured diversity and a
102 directional gene flow linked to human ditch cleaning. *P. coloratus* is protected in several
103 regions in France, such as in Alsace (Eastern France) where populations that are restricted to
104 small phreatic rivers have been reduced by half in 50 years (Carbiener *et al.*, 1990). In other
105 French regions, this species seems to occur in heterogeneous hydrosystems, even if its
106 ecology and distribution remains poorly studied. Buchwald et al. (1995) described three
107 different macrophyte communities comprising *P. coloratus*, postulating their differential
108 tolerance of eutrophication, and thus their distinct bioindicative values.

109 Aligning our study to the European frameworks for the enhancement of water
110 bioindication based on macrophytes and the conservation of a threatened aquatic species, we
111 here focus on French populations of *P. coloratus* with two main questions: (i) What are its
112 ecological niche and amplitude? Is it a strict stenoeceous species occurring only in
113 oligotrophic waters? For this goal, chemical and physical parameters of sites were compared
114 with aquatic plant and macroinvertebrate communities. (ii) Are genotypes correlated to
115 specific habitat types? Are there ecotypes within *P. coloratus* indicating different
116 environmental conditions? For this genotyping approach, we used AFLP multilocus
117 fingerprints and nrDNA ITS ribotypes.

118

119 **2. Materials and methods**

120 *2.1. Study sites*

121 In France, the calcareous affinity of *P. coloratus* mainly places it eastward, along the
122 Rhine – Rhône Quaternary basins, under alpine influence. Our study sampling focuses on
123 three eastern French hydro-ecoregions, (i) the Upper Rhine Plain (Rhine), (ii) the Upper
124 Rhône Valley and Northern Prealps (Rhône), and (iii) the French East-Mediterranean region
125 (Provence). In each one, we randomly sampled ten sites with *P. coloratus* (labelled AA-AJ,
126 RA-RH and PA-PJ, respectively; Fig. 1; Table 1; more details in Table S1, Appendix A),
127 based on species distribution data from SiFlore (Office Français de la Biodiversité,
128 <https://siflore.fcbn.fr>), Silene (Conservatoire Botanique National Méditerranéen,
129 <https://flore.silene.eu>), Taxa (Conservatoire Botanique d'Alsace) and Brunfels (Société
130 Botanique d'Alsace) databases. One site (PH) from Provence was dropped out because of the

131 local extinction of *P. coloratus* during the field period, between 2017 and 2018. Each site was
132 visited three times: one time in summer 2017 for genetic sampling, water sampling, and
133 macrophyte inventories, and two times in 2018, in spring and summer, for water and
134 invertebrate sampling.

135

136 2.2. *Biological sampling*

137 Macrophyte communities were identified exhaustively in lentic systems, and along a
138 100 m section in lotic systems. For the floristic analyses, we only kept macrophyte taxa
139 indicated in the IBMR (Haury et al., 2006). Species cover values in percentage were classified
140 into five cover categories according to the IBMR method and were then analysed in a
141 principal coordinates analysis (PCO) based on Bray-Curtis dissimilarities using *ade4* R-
142 package (Dray and Dufour, 2007) in R environment v.3.5 (R core Team 2020).

143 In each site, different microhabitats were sampled for macroinvertebrates using a sweep
144 net (40 × 40-cm frame net, 500-µm mesh size). Microhabitat types were sampled
145 independently for every site using a kick sampling protocol (Furse et al, 1981). Each site was
146 sampled following this protocol, by moving the net on each substrate and to kick it by foot
147 and sometimes by hand for 3-5 minutes proportionally to habitat representativeness. All
148 samples were preserved *in situ* in 90% alcohol. In the laboratory, samples were hand-sorted
149 and identified with a Nikon stereomicroscope (10-60x). Identification was mostly done to
150 genus level and sometimes to species level for some pollution-sensitive taxa such as
151 Ephemeroptera, Plecoptera and Trichoptera. Hydracarina, Nematoda and Oligochaeta were
152 identified as such. Data from spring and summer 2018 were pooled for the community
153 analysis.

154

155 2.3. *Water analysis*

156 Water temperature, pH, and conductivity were measured *in situ* using a multimeter
157 Multi 3620 IDS, WTW (Xylem Analytics, Weilheim, Germany). In the laboratory, chemical
158 parameters were measured using a high-performance liquid ion chromatography for cations
159 (Na^+ , NH_4^+ , Ca^{2+} , Mg^{2+} ; NF EN ISO 14911) and anions (Cl^- , NO_3^- , PO_4^{3-} , SO_4^{2-} ; NF EN ISO
160 10304). For the Total Kjeldahl Nitrogen (TKN), water samples were mineralised with
161 concentrated sulphuric acid (NF EN 25663) and boiled for distillation; then TKN was
162 estimated following the Nessler method. For the total phosphorus (TP), water samples were

163 oxidised with persulfate under acidic conditions for *ortho*-sulphate transformation; TP was
164 then estimated by molecular absorption spectroscopy (NF EN ISO 6878). For the total organic
165 and inorganic carbon (TOC and TIC), water samples were fully oxidized by combustion, and
166 CO₂ was estimated using the COTmeter 1030W Bioritech. All these parameters were
167 estimated and analysed for every site for the three sampling campaigns, except for TIC and
168 TOC which were only estimated once and reported for the three date in analyses.

169

170 2.4. DNA extraction and ITS sequencing

171 For genomic DNA extraction, ~ 50 mg of *P. coloratus* ground dried leaf material were
172 incubated 1 h at 65°C in 0.7 mL of cetyl-trimethyl-ammonium bromide (CTAB). An equal
173 volume of chloroform: isoamyl alcohol (24:1) was then added. After 30 s of vortex mixing,
174 samples were centrifuged for 10 min at 13000 rpm at room temperature. The aqueous phase
175 was transferred into new tubes with an equivalent volume of 0.6 M sodium acetate and 95%
176 ethanol [v/v], and gently mixed for DNA precipitation at -20°C overnight. DNA pellets were
177 centrifuged for 15 min at 13 000 rpm at 5°C, then washed in 70% ethanol, centrifuged again 5
178 min, dried and suspended in TE-buffer with RNase. DNA concentrations were measured
179 using a NanoDrop ND-1000 spectrophotometer (Labtech, Uckfield, UK), and diluted to
180 50 ng/μL in 1X TE-buffer. Internal transcribed spacers of ribosomal DNA were amplified in
181 PCR using the primers ITS5 (5'-GGAAGGAGAAGTCGTAACAAGG; Sang et al., 1995) and
182 ITS4 (5'-TCCTCCGCTTATTGATATGC; White et al., 1990) following the standard protocol
183 suggested by Promega (Madison, USA) for the GoTaq G2 Flexi DNA Polymerase. For one
184 sample in a final volume of 25 μL, 1X of GoTaq Flexi Buffer, 1 mM of MgCl₂, 0.2 mM of
185 dNTPs, 0.1 mM of each primer, 0.625 unit of polymerase, and 2.5 μL of template DNA at
186 50 ng/μL. Amplicons were then purified and sequenced by Eurofins Genomics
187 (Luxembourg). Two individuals were sequenced per site. Sequences were aligned, and
188 electrophoregrams were manually checked for nucleotide heterozygosity using MEGAX
189 (Kumar et al., 2018).

190

191 2.5. Amplified fragment length polymorphism (AFLP)

192 Ten individuals per site were sampled for a population genetic analysis. The AFLP
193 procedure was conducted according to Vos et al. (1995) and with the following modifications:
194 0.5 μg of DNA was digested with 10 units of *Eco*RI and 4 units of *Tru*9I in a final volume of

195 25 μ L, incubated during 3 h at 37°C and 3 h at 65°C. Ligation was performed at room
196 temperature for 8 h, by adding 0.5 unit of T4 DNA ligase, 12.5 mM of ATP, 3.125 units of
197 Eco adaptors and 31.25 units of Mse adaptors to the digested DNA. Preamplification was
198 performed with 0.2 ng ligation product, 1X of GoTaq Flexi Buffer, 0.25 μ M of Eco+A and
199 Mse+C primers, 1.0 mM of MgCl₂, 0.2 mM of dNTP and 0.625 unit of GoTaq G2 Flexi DNA
200 polymerase (Promega) in a final volume of 20 μ L. Preamplification cycles were performed at
201 94°C for 2 min, followed by 20 cycles at 94°C for 45 s, 56°C for 45 s, 72°C for 1 min and
202 72°C for 10 min. After testing several couple of primers, selective amplifications was
203 performed using Eco+AAC -- Mse+CAA, and Eco+ATC -- Mse+CCT primers dyed with 6-
204 FAM fluorescent at 5' end, Eco+AGC -- Mse+CGT primers dyed with ATTO550, and
205 Eco+AAT -- Mse+CAT primers dyed with ATTO565 (Eurofins Scientific, Luxembourg).
206 Selective amplification was performed in 20 μ L volumes with 5 μ L of 10 \times diluted
207 preamplification, 1X of GoTaq Flexi Buffer, 0.25 μ M of each primer, 1.0 mM of MgCl₂, 0.2
208 mM of dNTPs, 0.5 unit of GoTaq polymerase. The amplification thermocycle profile was as
209 follows: 94°C for 2 min, 10 cycles of 94°C for 30 s, 65°C for 30 s (-0.7°C touchdown per
210 cycle), 72°C for 1 min, followed by 20 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min
211 and 72°C for 5 min. Lengths of PCR products were obtained on an ABI Genetic Analyser
212 3130 (ThermoFischer Scientific, Waltham, MA, USA) at the IBMP genomic platform
213 (CNRS, Unistra). PCR product sizes were calibrated using the size standard GeneScan 600
214 LIZ v2.0 (ThermoFischer). Electrophoretograms were semi-automatically transformed in
215 presence-absence datasets using GeneMapper v.4.1 (Applied Biosystems, Thermo Fischer
216 Scientific, Waltham, USA). AFLP analysis was made by defining putative loci as any
217 fragment with a minimal amplitude of 200 relative fluorescent units (RFU) from 50 to 500 bp,
218 and automated scoring was manually checked to correct any misinterpreted RFU signal and to
219 discard any overlapping or ambiguous markers. Thirty replicate samples (one in each
220 population) were used to detect non-repeatable markers. We discarded every mismatching
221 marker between one sample and its replicate from the binary matrix of genotypes. Species
222 genetic structure and admixture was inferred based on individual ancestry coefficients using
223 the *snmf()* R-function implemented in the *LEA* R-package (Frichot & François, 2015) under R
224 environment. The choice of the most suitable number of ancestral populations (*K*) was based
225 on the cross-entropy criterion. The analysis of molecular variance was generated with the
226 *amova()* R function from the R package *pegas* (Paradis, 2010).

227

228 3. Results

229 *Water parameters*

230 Among the 29 sites, we generally found *P. coloratus* in relatively cold water, with a
231 median value of 14.5°C for the summer period. Sites had a low N-NH₄⁺ content with a
232 median value of 20 µg/L, and 90% of all values under 100 µg/L (Appendix A, Figs. S1 & S2).
233 As expected, *P. coloratus* was found in hard water, with Ca and Mg mean concentration of
234 80.4 ± 33.0 mg/L and 33.6 ± 15.2 mg/L, respectively. Physical and chemical parameters of
235 water samples showed a strong regional pattern: water from the Rhine Plain showed the
236 highest carbon content (TOC and TIC) and nitrogen-nitrate (N-NO₃⁻: 7.0 ± 1.5 mg/L for the
237 Rhine vs. 3.7 ± 3.2 mg/L globally), but the lowest temperature (13.2 ± 1.2°C for the Rhine vs.
238 15.8 ± 4.1°C globally). The content in P-PO₄³⁻ was nearly equivalent between regions, with a
239 mean of 90.6 µg/L. Water from the Rhone Valley was slightly more acidic and softer, with
240 lower Ca, Mg, Na and Cl contents. Water from Provence showed the widest variability
241 because of the heterogeneity of catchments sampled in this region, contrary to the Rhine and
242 the Rhone regions. The tree samples per site showed a weak variation of water parameters
243 within each site. The percentage of cover of *P. coloratus* in each site was not significantly
244 correlated with any abiotic variable, except a small negative correlation with pH (Pearson
245 correlation = -0.47; P = 0.010).

246

247 *Plant and invertebrate communities*

248 The plant diversity of the 29 communities including *P. coloratus* was estimated to range
249 from 3 to 17 with a mean value of 7.7 ± 3.3. The main species co-occurring with *P. coloratus*
250 were *Mentha aquatica* (19 sites), *Berula erecta* (13), *Phragmites australis* (13), *Juncus*
251 *subnodulosus* (9) and *Batrachospermum* sp. (7). The ordination analyses of macrophyte
252 communities showed a major gradient based on the PCO first axis including 25.4% of floristic
253 dissimilarities (Fig. 2A). This distinguished species-poor communities to the left (species
254 richness < 7) including *Berula erecta* and *Mentha aquatica* and mainly represented in the
255 Rhine Plain, to more diversified communities to the right (species richness > 7) mainly
256 occurring in the Rhone Valley and in Provence.

257 We identified 164 taxa of macroinvertebrates across the 29 sites, mainly at the genus
258 level. The mean macroinvertebrate taxonomic richness per site was 30.2 ± 8.7, with a
259 minimum of 13 and a maximum of 49. All together, we observed a relatively higher diversity

260 of Coleoptera (32 genera), Trichoptera (25 genera), Gastropoda (22 genera) and Odonota (20
261 genera) compared to Ephemeroptera (11 genera) and Plecoptera (one genus). Besides the
262 ubiquity of different Chironomidae tribes in the 29 sites, the most common taxa were
263 *Gammarus* sp. (25 sites), *Sialis* sp. (20 sites) and Oligochaeta (20 sites). The ordination
264 analysis of invertebrate communities showed a major gradient linked with species diversity
265 and feeding habits (Fig. 2A): diversified communities with an abundance of predators are at
266 the bottom of the PCO#1 axis, and less diversified communities dominated by shredders at
267 the top of the axis.

268 Analysed together, the macrophytes and macroinvertebrates PCO first axes show a
269 strong negative correlation (Pearson correlation = -0.66; p-value < 0.001). They distinguished
270 lotic from lentic hydrosystems (Fig. 2A). The projection of abiotic parameters on these PCO
271 axes mainly explains these two ecological groups with the negative correlation of water
272 velocity and temperature (Fig. 2B). As an index of species performance, the percentage of
273 cover of *P. coloratus* does not seem to be correlated with environmental gradients (biotic
274 PCO axes; Fig. 2C).

275

276 *Genetic differentiation*

277 The 736-bp alignment of nrDNA ITS for the 58 individuals allowed us to distinguish
278 two ribotypes, based on four substitution sites supporting the same clustering. The ribotype 1
279 (GenBank accession number: MW662121; herbarium specimen STR031810) was found in 17
280 populations, and is the only haplotype found in the Rhine Plain; the haplotype 2 (GenBank:
281 MW662122; herbarium specimen STR031811) was found in 12 populations. After
282 examination of electrophoretograms, only one individual from the PB population (Fos,
283 Provence) shows heterozygosity, with the overlap of the ribotype 1 and the ribotype 2. A
284 BLASTn search clearly associated these two ribotypes to *P. coloratus* sequences already
285 deposited in GenBank. The other individuals of the same population possessed only the
286 haplotype 1. This ribotype clustering did not show any correlation with the main habitat
287 differentiation previously described.

288 The AFLP protocol based on 261 individuals generated 343 markers after discarding
289 of non-repeatable ones (error rate of 2.4%), of which 41% were polymorphic. However,
290 polymorphism decreased to 5% on average for any given population. The AMOVA described
291 10% of the genetic variability between the three regions, and 59% between populations.

292 Population gene diversity was relatively low, with a mean of 0.016. Regional gene diversity
293 was two times lower in the Rhine Plain (0.024) than in the two southward regions (0.050 in
294 Provence and 0.061 in Rhone). The admixture structure analysis distinguished two main
295 ancestral populations based on the cross-entropy criterion, with only five more or less
296 admixed populations (AD, PA, PB, RB, RI; fig. 3). Two main lineages were distinguished:
297 the first one representing the majority of the sampling, and the only one occurring in the
298 Rhine Plain (except the slightly admixed AD; fig. 3). The second lineage was dominant only
299 in three populations (PC, RF and RG; fig. 3) but did not occur as non-admixed genotypes in
300 the Rhine Plain. The neighbour-joining tree based on AFLP distances discriminated most of
301 the populations, without clear regional differentiation between Provence and Rhone, and with
302 the gathering of almost all populations of the Rhine in a same lineage (Appendix A, Fig. S3).
303 Interestingly, only the AD Rhine population was not included in this Rhine lineage, but rather
304 shares genetic proximity with PF (Nice, Provence: Appendix A, Fig. S3). We did not find any
305 correlation between AFLP lineages and habitat differentiation.

306

307 **4. Discussion**

308 4.1. A diversity of habitats with some common features

309 The present study was initially started to understand why *P. coloratus* was threatened
310 and restricted to phreatic waters in the Rhine Plain, contrary to other French regions. Indeed,
311 our field prospections found only 10 populations in the Rhine Plain, exclusively in small
312 phreatic watercourses and springs (Table S1, Appendix A). In the Rhône Valley and
313 Provence, where its ecological distribution was less documented, we also found it in several
314 hydrosystems with putative phreatic inflow. In Provence, the most evident ones were two
315 springs, but we also found the species in two phreatic watercourses (named 'Laurons' in the
316 South of the Crau Plain; Giudicelli et al., 1980). In the Rhone Valley, we also sampled one
317 spring, and six hydrosystems (ponds, oxbows named 'Lônes', and secondary canals) fed by the
318 aquifer of a large river such as the Ain and the Rhône rivers. The main marker of this phreatic
319 influence remains the low water temperature of these hydrosystems. Biotic (macrophytes and
320 macroinvertebrates) and abiotic (water parameters) characteristics both distinguish *P.*
321 *coloratus* in two main groups of habitats: (i) the lotic ones, including springs and small
322 watercourses; and (ii) the lentic ones, with shallow ponds and oxbows. This classical
323 distinction in hydrobiology could however be mitigated by some similarities. The water of the
324 29 study sites was mostly neutral to base-rich (mean pH 7.5 ± 0.4) and cool (mean

325 temperature $15.8 \pm 4.1^{\circ}\text{C}$). These small and relatively stable habitats were previously
326 described as the ecological optimum of *P. coloratus* in the literature (Buchwald et al., 1995;
327 Hollingsworth et al., 1995; Preston, 1995; Gornall et al., 1998; Kaplan, 2010). The species
328 was also described as a pioneer species, poorly competitive and appearing in early stages of
329 succession of shallow wetlands (e.g. after an extensive disturbance) and then disappearing in
330 later successional stages predominated by dense marshland vegetation (Buchwald et al.,
331 1995). During field prospection in Provence, we also temporally observed *P. coloratus* on
332 shallow margins or in ponds within point bars of large rivers (such as the Durance) before
333 annual floods. this type of unstable and temporary habitats is underrepresented in the present
334 study. Another important perturbation that leads to its local extinction is the partial or
335 complete drying of its habitat, often linked to a lowering water table. This permanent drying
336 could explain about half of local extinctions observed during our field prospection in the
337 Rhine Plain.

338

339 4.2. *Potamogeton coloratus*, a bioindicator of oligotrophic waters?

340 In European methods assessing river trophic levels based macrophyte communities,
341 *P. coloratus* often possesses one of the most oligotrophic contribution (e.g. Schneider and
342 Melzer, 2003; Haury et al., 2006). In particular, its high sensitivity to ammonium is often
343 cited (Trémolières, 2004), based on old laboratory tests (Glänzer, 1974; Kohler, 1975;
344 Glänzer et al., 1977) whose results' interpretation was questioned (Demars and Edwards,
345 2009). Instead, Schneider and Melzer (2004) targeted phosphorus as a restraining factor for *P.*
346 *coloratus*. Our study also found this species in low ammonium concentration, with most of
347 sites under $50 \mu\text{g/L N-NH}_4^+$, and in low temperature (mean 15.8°C in summer). Occurrence in
348 low water temperatures is poorly documented in the literature, but Bayindir and İkinci (2019)
349 recently described *P. coloratus* as the pondweed occurring in the coldest hydrosystems, with a
350 mean of $14.2 \pm 2.8^{\circ}\text{C}$, and tolerating the lowest temperature range. Our results also
351 confirmed the relatively poor sensitivity of *P. coloratus* to variations of nitrate and phosphate,
352 as suggested before (Tremolières and Hardion, 2017). An unexpected result was to find
353 significantly higher nitrate concentrations in the Rhine Plain, where the species is protected
354 and emblematic of species-poor communities in oligotrophic waters. Thirty years ago,
355 phosphate and nitrate concentrations were respectively 10 and 4 times lower in the
356 watercourses sampled in the Rhine Plain (Carbiener et al., 1990). This paradox between

357 higher nitrate contents but still species-poor communities must be put in perspective with
358 other water parameters, such as temperature, which is 3°C lower in the Rhine Plain. This cold
359 and stable temperature over the year could explain the limited competition and successional
360 stages for Rhine phreatic watercourses hosting *P. coloratus*, despite increasing nutrient
361 concentrations. This differential species sensitivity to water parameters reaffirms the
362 multispecies dimension of water bioindication as a global index giving a simplified view of
363 river trophic level, but not as an accurate tool estimating nutrient concentrations (Demars et
364 al., 2012). In this way, the French macrophyte bioindicator system IBMR informs us about a
365 macrophytes' response to the global abiotic context: eutrophication can happen not only if
366 nutrient concentrations are in excess, but also if all other environmental parameters allow
367 vegetation growth (e.g. light, temperature). For a detailed interpretation of species occurrence,
368 laboratory experiments in a range of water parameter concentrations would be necessary. In
369 such experiments, it would be interesting to couple different parameters, for example varying
370 degrees of temperature and a range of nutrient concentrations, and to evaluate the effect of
371 different response variables on *P. coloratus*. Finally, the present study only analysed water
372 parameters, neglecting sediment parameters. This could be relevant, as *Potamogeton*
373 *coloratus* is a rhizomatous species for which such environmental factors could be prominent.
374 Indeed, Schneider and Melzer (2004) also found *P. coloratus* in nutrient poor waters among
375 Bavarian rivers compared to other macrophyte species. However, the species was rooted in
376 sediments with the highest N and C total contents, and medium ammonium concentrations
377 (median exceeding 2 mg/L N- NH₄⁺).

378

379 4.3. A low genetic diversity, without genotype-ecotype correlation

380 Aquatic plant species are known for their high clonality (Silvertown, 2008) and their
381 low within-species genetic diversity (Santamaría, 2002). However, numerous population
382 genetics studies have softened this axiom (e.g. Li et al., 2004; Jiang et al., 2020). In particular,
383 low within-population genetic diversity vs. high between-population genetic differentiation is
384 a common pattern for macrophytes (e.g. Islam et al., 2020). Our study partially follows this
385 scenario, with nearly null diversity within most populations of *P. coloratus*, and relatively low
386 genetic diversity within regions despite genetic differentiation between populations. However,
387 these results contrast with the previous studies on *P. coloratus* highlighting genetic diversity
388 and structure in South West England based on isozyme variation (Hollingsworth et al., 1995;

389 Gornall et al., 1998). These authors suggested a possible Pleistocene persistence of the species
390 in England, despite little evidence in glacial deposits. Unfortunately, *P. coloratus* has not
391 been studied in this country since then with more robust genetic markers, and neither in other
392 European countries. This poor species genetic diversity should be softened considering the
393 reduced sampling area of the present study. Indeed, we collected this species in less than half
394 of its distribution, neglecting regions where the species seems the most abundant, such as
395 British Islands, Spain and the South of Scandinavia.

396 The two putative ecotypes (lotic and lentic) neither match the two main genetic
397 clusters delineated by AFLP markers nor the two ITS ribotypes. The identification of ecotypic
398 distinction supported by genotypic differentiation remains rare for macrophytes, and often
399 related to factors that constrain clonal reproduction, as salinity and ephemeral inundation
400 (Santamaría, 2002). To date, one of the best examples is represented by the ecotypes-
401 genotypes of *Stuckenia pectinata* differentiated along a salinity gradient (Nies and Reusch,
402 2005; Triest et al., 2010). In our study, the geographical proximity between populations is
403 often correlated with a genetic similarity, except for two populations (RC and RD) from the
404 Rhone Valley. These two sites located a hundred meters apart, but in distinct ecological
405 conditions (lotic vs. lentic) showed a slight genetic differentiation (Appendix A, fig. S3). This
406 consistent genetic difference could be due to an ecological differentiation, a limited gene flow
407 and/or a recent event of dispersal. One of the most interesting results from our genetic dataset
408 is the occurrence of a putative long-distance dispersal in the Rhine Plain. Indeed, the region
409 showed deeply similar genotypes and nearly null genetic variation. However, one population
410 (AD) is genetically closer to a population from Provence (PF) than it is to those in the Rhine,
411 while the abiotic conditions of its site are not different from other regional sites. This
412 population was observed for the first time in the 2000s, despite early prospections and studies
413 on the regional distribution of *P. coloratus* (Klein and Carbiener, 1989; Buchwald et al.,
414 1995). This relatively recent finding of a differentiated genotype could reflect the ability of
415 this species for long distance dispersal, as recently suggested by Lukács et al. (2020).

416

417 4.4. Insights for species conservation

418 A slight paradox emerged from the literature between the description of *P. coloratus*
419 as a pioneer species (Buchwald et al., 1995), distributed in drainage ditches and resilient to

420 ditch cleaning (Gornall et al., 1998), on one side, and a sensitive oligotrophic species
421 distributed in stable environments on the other side (Lukács et al., 2020). Mony et al. (2011)
422 have also demonstrated its low vegetative growth and its poor resilience to perturbations. Our
423 study confirms that *P. coloratus* mainly occurs in relatively cold water with low ammonium
424 concentrations. Obviously, these preferences are not strict, and *P. coloratus* is not a precise
425 bioindicator species of oligotrophic waters, nor are any of its genotypes. In other words,
426 *P. coloratus* seems to be amongst the macrophytes tolerating the lowest temperatures and
427 nutrient concentrations in the field. In addition, this species is very sensitive to variations in
428 water levels (Lukács et al., 2020), and even more so to the drying of rivers which is the main
429 cause of population extinction in the Rhine Plain. The species was extinct but then
430 rediscovered in the Netherlands, Czech Republic and Hungary: In the Netherlands and Czech
431 Republic, the reappearance was attributed to its persistence in the seed bank in previously
432 disturbed sites (Bruin, 1997; Kaplan et al., 2014). However, in Hungary, Lukács et al. (2020)
433 also designated avifauna as an effective vector for viable seed dispersal. Indeed, they
434 demonstrated that seeds of *P. coloratus* force-fed to mallards passed through their digestive
435 tract and remained viable. The ability for long distance dispersal, also suggested in our study,
436 could explain the temporary occurrence of *P. coloratus* in post-disturbance ecosystems
437 mentioned above.

438 For conservation purposes, undisturbed hydrosystems with sufficient water supply, and
439 low temperatures and ammonium contents should be a priority habitat for species
440 conservation. Recent species colonisation of post-disturbance habitats could be less pertinent
441 for long-term species persistence (Hollingsworth et al., 1995). In the Rhine Plain, if the
442 increasing water table nutrient content is effective and alarming, other parameters could also
443 explain population extinctions, separately or interactively. More specifically, we suggest
444 testing the following ones: the drying-up of rivers due to a lowered water table combined with
445 higher water temperatures due to global warming, and light limitation due to the closing-up of
446 riparian arboreal vegetation.

447

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455

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458

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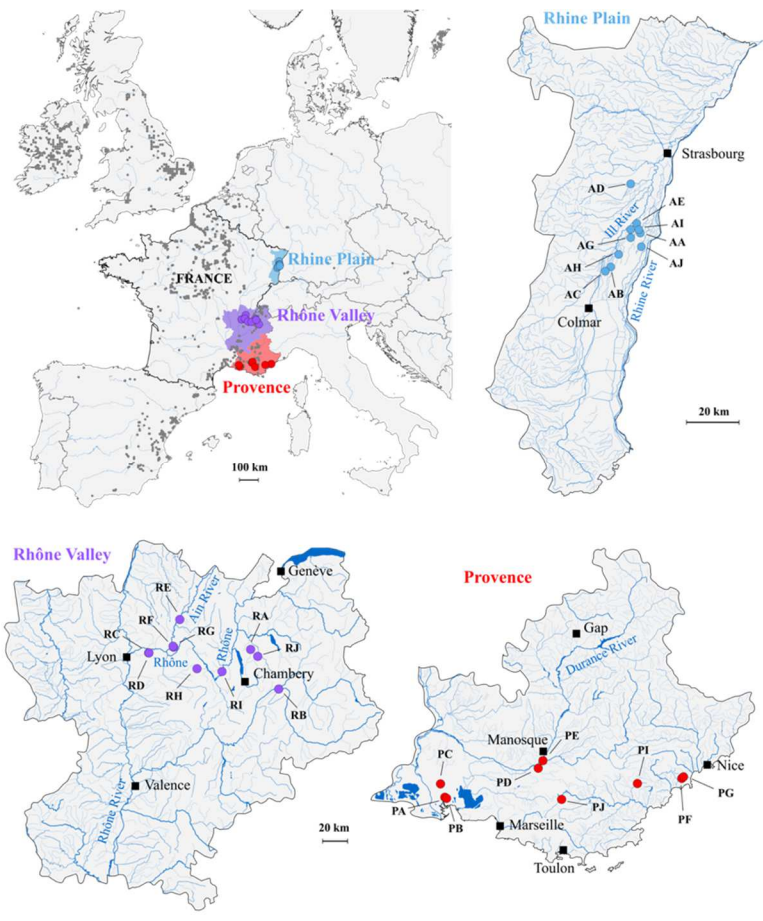
597 **Table 1.** sampling localities, hydrosystem types, mean water parameters, species richness for
598 macro-invertebrates (S inv) and macrophytes (S veg), AFLP genotypes and ITS ribotypes.

Site	Locality	Region	System	Type	T°	O2	Conduc	N-NH4	N-NO3	P-PO4	SO4	S inv	S veg	AFLP	ITS
PA	Fos-sur-mer	Provence	Ditch	lotic	17.2	7.8	723	0.04	3.4	0.05	97.2	40	8	G1	ribo2
PB	Fos-sur-mer	Provence	Pool	lentic	21.3	16.8	466	0.02	0.6	0.07	113.9	18	9	G1	ribo1
PC	St-Martin-de-Crau	Provence	Canal	lotic	17.5	9.2	722	0.01	2.4	0.02	127.8	22	10	G2	ribo2
PD	St-Paul-les-Durance	Provence	Spring	lentic	14.2	7.1	659	0.05	3	0.37	25.1	13	8	G1	ribo1
PE	Vinon-sur-Verdon	Provence	Stream	lotic	12.1	8	453	0.11	1.2	0.03	33.3	31	9	G1	ribo2
PF	Valbonne	Provence	Pool	lentic	17.2	3.3	429	0.07	0	0.02	5.3	16	12	G1	ribo2
PG	Biot	Provence	Stream	lotic	18.7	11	763	0.02	2	0.06	57.7	39	13	G1	ribo2
PI	Fayence	Provence	Stream	lentic	17.2	8.6	987	0.01	0.1	0.08	312.8	34	10	G1	ribo1
PJ	Bras	Provence	Spring	lentic	14.6	6.2	807	0.01	6.1	0.08	138.1	45	10	G1	ribo1
RA	Braille	Rhone Valley	Spring	lentic	13.2	3.5	505	0.09	2.5	0.04	5.8	23	5	G1	ribo2
RB	Bourgneuf	Rhone Valley	Pond	lentic	24.8	5.3	605	0.07	0.3	0.1	70.6	29	6	G1	ribo2
RC	Meyzieu	Rhone Valley	Canal	lotic	18.9	3.1	365	0.01	0.7	0.07	35.6	31	9	G1	ribo1
RD	Meyzieu	Rhone Valley	Pool	lentic	19.7	1.4	421	0.02	0.1	0.09	13.4	29	17	G1	ribo2
RE	Priay	Rhone Valley	Oxbow	lentic	18	6.8	475	0.01	2.3	0.09	8.4	46	9	G1	ribo2
RF	Ricoti	Rhone Valley	Oxbow	lotic	19.9	9.6	604	0.09	7.1	0.13	20.1	36	6	G2	ribo2

RG	Gourdan	Rhone Valley	Owbow	lentic	18.5	6.3	441	0.04	0.7	0.06	8.9	49	13	G2	ribo2
RH	Morestel	Rhone Valley	Canal	lotic	15.2	6.3	604	0.06	4	0.19	20.3	25	5	G1	ribo1
RI	La-Balme	Rhone Valley	Canal	lotic	12.8	4.7	402	0.13	1.4	0.08	33.7	24	4	G1	ribo1
RJ	Cusy	Rhone Valley	Ditch	lotic	16.6	7	288	0.09	0.3	0.04	2.6	31	6	G1	ribo2
AA	Herbsheim	Rhine Plain	Spring	lotic	12	5	601	0.02	5.7	0.18	38.8	27	5	G1	ribo1
AB	Selestat	Rhine Plain	Stream	lotic	13.7	9.1	957	0.03	8.7	0.04	45.6	29	9	G1	ribo1
AC	Guemar	Rhine Plain	Stream	lotic	13.6	7.2	930	0.02	6.9	0.03	38.4	43	3	G1	ribo1
AD	Innenheim	Rhine Plain	Stream	lotic	14	4.8	746	0.09	7.2	0.2	103.3	31	5	G1	ribo1
AE	Sand	Rhine Plain	Spring	lotic	11.7	4.9	646	0.01	7.6	0.19	40	24	3	G1	ribo1
AF	Huttenheim	Rhine Plain	Ditch	lotic	12.5	6.1	668	0.02	6.8	0.04	42.3	25	5	G1	ribo1
AG	Witternheim	Rhine Plain	Stream	lotic	12.8	7.6	650	0.02	8.5	0.02	39.1	31	6	G1	ribo1
AH	Muttersholtz	Rhine Plain	Stream	lotic	13.5	8.1	668	0.14	8.2	0.08	43	27	5	G1	ribo1
AI	Herbsheim	Rhine Plain	Stream	lotic	14	10.2	623	0.02	6.3	0.07	38.4	30	6	G1	ribo1
AJ	Diebolsheim	Rhine Plain	Ditch	lotic	14.3	7.2	642	0.03	4.4	0.12	41	29	6	G1	ribo1

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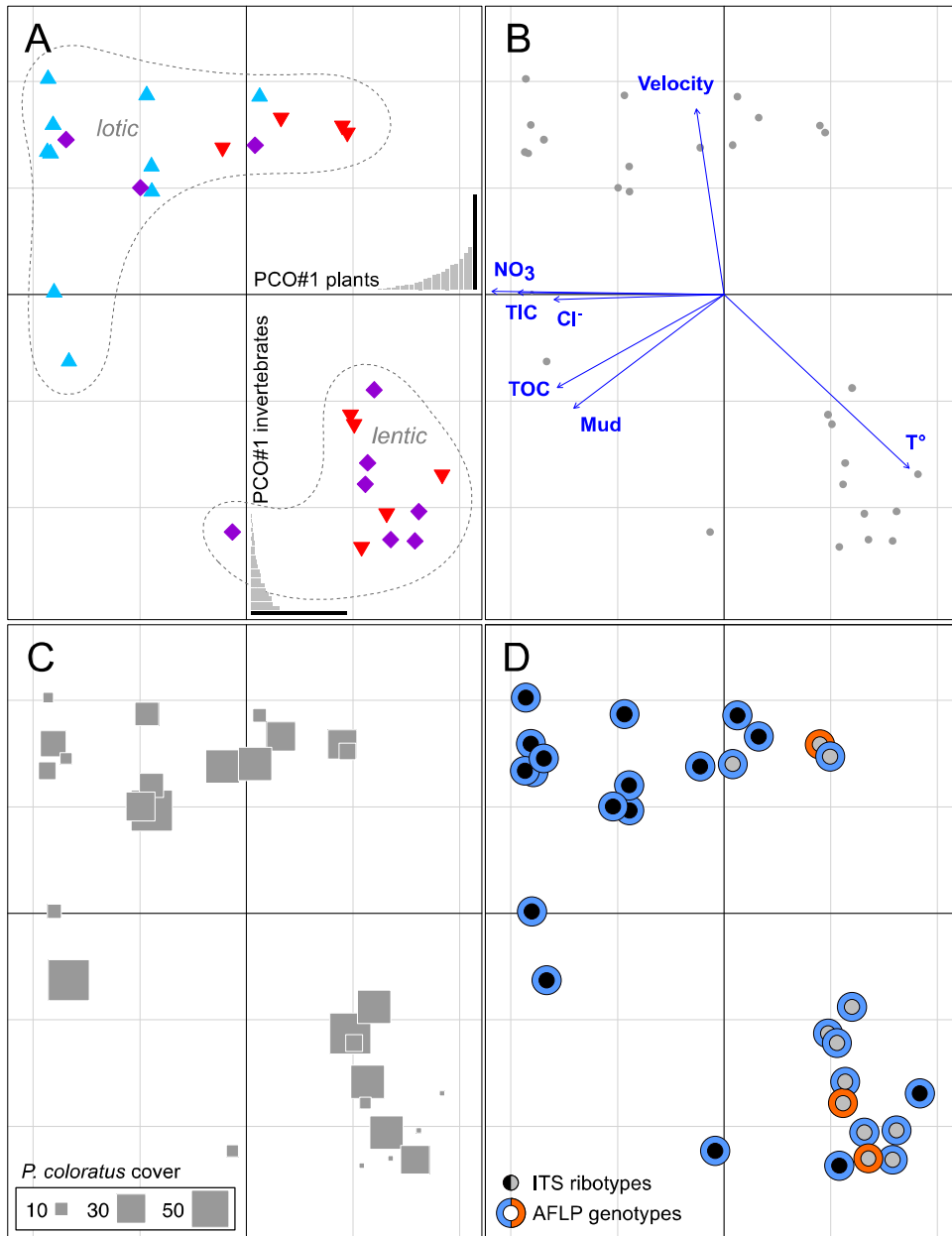
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603 **Figure 1.** Distribution of *Potamogeton coloratus* in Europe (grey dots; sources: GBIF for
 604 Europe, www.gbif.org; FCBN-OFB for France, https://siflore.fcbn.fr) and sampling sites in
 605 the three French ecoregions.

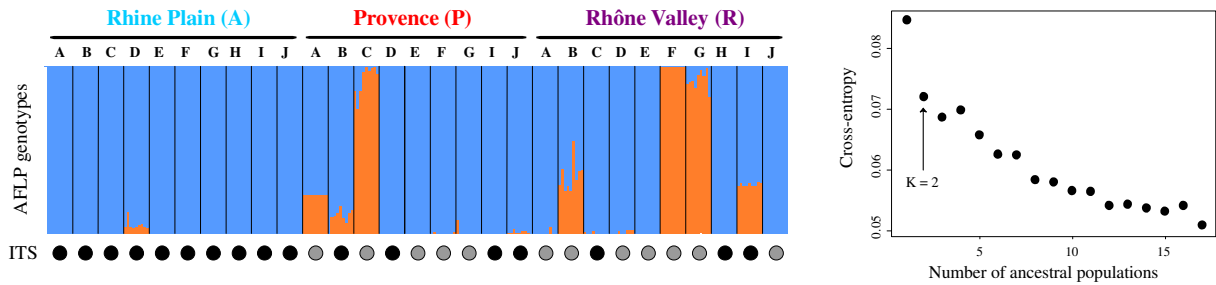


606 **Figure 2.** A) ecological differentiation of the 29 sites of *Potamogeton coloratus* from
 607 Provence (red triangles), Rhone (purple squares) and Rhine (blue triangles) ecoregions based
 608 on the plant PCO first axis (25.4% of plant dataset variability) and on the invertebrate PCO
 609 first axis (35.6% of the invertebrate dataset variability). The two PCO axes were significantly
 610 correlated (Pearson's correlation = -0.662, p -value < 0.001), distinguishing lotic from lentic
 611 habitats. B) same biplot with fitting abiotic environmental variables significantly correlated
 612 with PCO axes ($P < 0.5$). C) same biplot with *P. coloratus* cover (%) on each site as an index
 613

614 of species performance. D) same biplot with genetic clusters based on ITS ribotypes and
615 AFLP genotypes, showing the absence of genotype-ecotype correlation.

616

617



619 **Figure 3.** Admixture clustering analysis of the AFLP dataset based on 261 individuals and
620 343 markers. The cross-entropy stall indicates that $K = 2$ is a suitable clustering number (i.e.
621 number ancestry coefficients) for the AFLP dataset, using the `snmf()` R-function implemented
622 in the *LEA* R-package (Frichot & François, 2015). The black and grey dots correspond to the
623 rDNA ITS ribotypes 1 and 2, respectively.

624

625

626 **Figure colors online only**