

1 **Reintroduced native *Populus nigra* in restored floodplain reduces**
2 **spread of exotic poplar species**

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24 **1. Abstract**

25 Exotic *Populus* taxa pose a threat to the success of riparian forest restoration in floodplain
26 areas. We evaluated the impact of exotic *Populus* taxa on softwood riparian forest
27 development along the river Common Meuse after introducing native *Populus nigra* and after
28 the re-establishment of the natural river dynamics. We sampled 154 poplar seedlings that
29 spontaneously colonised restored habitat and assessed their taxonomy based on diagnostic
30 chloroplast and nuclear microsatellite markers. Furthermore, by using a paternity analysis on
31 72 seedlings resulting from six open pollinated *P. nigra* females, we investigated natural
32 hybridization between frequently planted cultivated poplars and native *P. nigra*. The majority
33 of the poplar seedlings from the gravel banks analyzed were identified as *P. nigra*; only 2%
34 of the sampled seedlings exhibited genes of exotic poplar species. Similarly, the majority of
35 the seedlings from the open pollinated progenies were identified as *P. nigra*. For three
36 seedlings (4%), paternity was assigned to a cultivar of *P. x canadensis*. Almost two decades
37 after reintroducing *P. nigra*, the constitution of the seed and pollen pools changed in the study
38 area in favour of reproduction of the native species and at the expense of the exotic poplar
39 species. This study indicates that, although significant gene flow from exotic poplars is
40 observed in European floodplains, restoration programmes of the native *P. nigra* can
41 vigorously outcompete the exotic gene flows and strongly reduce the impact of exotic
42 *Populus* taxa on the softwood riparian forest development.

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44 **Key-words:** European black poplar; floodplain ecosystem; hybridization; interspecific
45 mating.; introgression; riparian forests

46

47 2. Introduction

48 The restoration and protection of riparian forests is one of the key priorities in biodiversity
49 conservation and climate change adaptation strategies (e.g. EU Biodiversity Strategy 2030,
50 EU Floods Directive 2007/60/EC). Riparian forests are biodiversity hotspots and provide a
51 range of ecosystem services including flood protection, prevention of bank erosion, thermal
52 regulation by forest canopy cover and water quality protection (e.g. Van Looy et al. 2013).
53 They are therefore necessary for the ecological functioning of riparian corridors and are
54 recognized as an important part of the world's natural capital.

55 After decades of ecological degradation caused by human activities, including impoundment
56 and straightening of rivers, cutting off meanders, drainage of floodplains and deforestation for
57 agriculture and plantations, several European countries have undertaken actions to restore
58 natural river dynamics (e.g. Mansourian et al. 2019; Schindler et al. 2016), and the European
59 Commission proposed a Biodiversity Strategy planning to restore at least 25,000 km of free-
60 flowing rivers (UE COM Biodiversity Strategy 20/05/2020). Generally, restoration activities
61 involve dyke relocation, lowering of river banks and floodplains to restore the floodplain
62 habitat heterogeneity, an essential feature for the regeneration of riparian forests. However,
63 many restoration projects are poorly documented and written records on the outcome of
64 restoration projects are generally lacking (Palmer et al. 2007).

65 Revegetation of river margins is a widely applied technique for riparian forest restoration
66 (Palmer et al. 2007). However, exotic, invasive alien species can significantly undermine
67 efforts to protect and restore natural floodplain forests (Palmer et al. 2007). Exotic *Populus*
68 species have been introduced into Europe as well as into the US and Canada for wood
69 production in riparian landscapes and pose a threat to native *Populus* species. Poplar cultivars
70 are frequently planted in monoclonal plantations in the vicinity of wild relatives and may
71 contribute to a large extent to pollen and seed pools, thereby competing with the native
72 relatives in colonizing restored habitat. Furthermore, intercrossability among *Populus* species
73 is well-known (Eckenwalder 1984, 1996). In Europe, the native European black poplar
74 (*Populus nigra* L.) (hereafter referred to as black poplar) is threatened by Euramerican (*P.* ×
75 *canadensis* Moench.), interamerican (*P.* × *generosa* Henry) hybrid poplar and black poplar
76 varieties such as the male Lombardy poplar (*Populus nigra* cv. *Italica* Du Roi) (e.g. Arens et
77 al. 1998; Cagelli and Lefèvre 1995; Chenault et al. 2011; Debeljak et al. 2015; Fossati et al.
78 2003; Ziegenhagen et al. 2008). Gene flow between cultivated and native poplar species may
79 result in the reduction of the effective population size, genetic swamping and ultimately the
80 replacement of populations of the native species (Arnold et al. 2001).

81 As a pioneer species, black poplar plays a key-role in the development of softwood forests in
82 Europe. In the lower parts of the floodplain, black poplar colonises bare moist soil and river
83 banks and is highly adapted to water dynamics and sediment movement (Debeljak et al. 2015;
84 Van Looy 2006). Due to their rapid growth and strong flow resistance, black poplar together
85 with species of *Salicaceae* (*Salix alba* and *S. viminalis*), contributes to raising bar and island
86 levels by retaining sand and gravel (Van Looy 2006). Consequently, these softwood forest
87 species pave the way for the development of the next forest succession stage, the hardwood
88 forests. Unfortunately, the European black poplar is one of the most threatened tree species in
89 Europe, mainly because of the loss of its natural alluvial habitats especially sand and gravel
90 banks that allow for successful reproduction, and because of competition and introgression

91 with exotic poplar species (Fossati et al. 2003; Meyer et al. 2018; Vanden Broeck et al. 2005;
92 Ziegenhagen et al. 2008).

93 Here, we report on the impact of exotic *Populus* taxa at the initial stages of softwood riparian
94 forest development of the river Meuse on the Dutch-Belgian border almost two decades after
95 introducing black poplar and after the re-establishment of the natural river dynamics. To our
96 knowledge, this is the first study reporting on the effect of re-introducing a native *Populus*
97 species on gene flow of exotic relative species. The specific objectives of this study are (i) to
98 study the taxonomy of the poplar seedlings that spontaneously colonised the river banks of the
99 Common Meuse and (ii) to determine the frequency of natural hybridization events between
100 male cultivated poplars (including *P. nigra* cv. *Italica*) and the female native black poplars in
101 the study area. We hereto use a combination of diagnostic chloroplast and nuclear molecular
102 markers that have been proven useful in identifying the taxonomy of *Populus* seedlings
103 (Heinze 1998; Imbert and Lefèvre 2003; Ziegenhagen et al. 2008). We compare the results
104 with a similar study performed in 1999 -2001 at the same site and before the restoration
105 actions took place (Vanden Broeck et al. 2004). Finally, we discuss the implications for the
106 restoration and conservation of softwood riparian forests with black poplar along European
107 rivers.

108 **3. Material & Methods**

109 3.1 Study site

110 The area of interest was the riverside of the Common Meuse, a free-flowing middle course of
111 the river Meuse, which forms for 45 km the border between Belgium and the Netherlands
112 (Figure 1). Shipping and flow regulation are absent on this river stretch. The Common Meuse
113 is a rain-fed river with a gravel-bed, a strong longitudinal gradient (0.45 m/km) and a wide
114 alluvial plain (Van Looy et al. 2005). The Common Meuse valley consists of a gravel
115 underground with a loamy alluvial cover. Traditionally, meadows were created in the
116 floodplains. Cultivated poplars are frequently planted at the study site (Vanden Broeck et al.
117 2004). Large parts of the alluvial plain have been excavated for gravel mining, leaving large
118 gravel pits or lowered floodplain zones (Van Looy et al. 2005). The extreme high water levels
119 and floodings of 1993 and 1995, were the start of the development of a large scale,
120 transboundary river restoration project for the Common Meuse; called 'Living River
121 Strategy'. The concept of this plan was to restore hydrodynamics and morphodynamics and
122 related ecological characteristics in the primary river channel (Van Looy et al. 2006). The
123 restoration activities were performed during the period 2005-2008 and included removal of
124 dykes, excavation of the floodplain, channel widening and bank lowering. The restoration of
125 the morphological activity (erosion / sedimentation rates) of the river reach resulted in a
126 strong revitalisation of the elevation of bars and islands, creating suitable habitat for seedling
127 recruitment and the restoration of softwood riparian forest with willow, poplar and ash
128 communities (Van Braeckel 2007; Van Looy et al. 2008).

129 3.2 Reinforcement of the Black poplar population

130 A reintroduction project of *P. nigra* was performed in the period 2002-2005. The aim was to
131 create seed sources and thereby to promote the colonisation of native *P. nigra* in newly
132 restored habitat. This was the follow-up of a study performed in 1999-2000 on the restoration
133 potential and constraints of the development on softwood riparian forests along the Common

134 Meuse (Vanden Broeck and Jochems 2002; Vanden Broeck et al. 2004). This former study
135 emphasized the need for re-planting native *P. nigra* since natural populations were almost
136 gone extinct in the study area and > 150 km further upstream in Northeast France (Vanden
137 Broeck 2004). A few isolated relict trees of *P. nigra* in the study area suggested the presence
138 of natural populations in the past. In contrast to the native poplar species, plantations of exotic
139 poplars of *P. x canadensis* and *P. nigra* cv. *Italica* were common within the study area
140 (Vanden Broeck et al. 2004).

141 *P. nigra* was successfully planted on two locations; location Groeskens (Dilsen-Stokkem,
142 51°01'01N, 05°46'02E) and location Negenoord (Dilsen-Stokkem, 51°01'36 N, 05°46'16E)
143 on 0.77 ha and 2 ha, respectively. In total, 275 two-year old bare-root *P. nigra* trees were
144 planted, with plant distances of 9 m x 9 m and 4 m x 8 m, in Groeskens and Negenoord,
145 respectively. The distance from the centre of the plantings to the river bank was about 140 m
146 for Groeskens and 5 m for Negenoord. The selection of the plant locations was based on the
147 close connection to the river and on locations indicated close to potential sites for riparian
148 forest development by a habitat model developed for the Common Meuse by Van Looy et al.
149 (2005). The plant material originated from the national *P. nigra* gene bank collections at the
150 Research Institute for Nature and Forest (INBO, Belgium; 57 genets) and at Alterra (The
151 Netherlands; 21 genets) supplemented with 23 genets from a natural population located along
152 the river Rhine in Germany (location Kühkopf, FA Groß-Gerau, 48° 48' 56,62 N, 8° 7' 38,60
153 O) and about 90 seedlings of controlled crosses performed at INBO with genotypes of *P.*
154 *nigra* from the gene bank collection. All the plant material, was identified as pure *P. nigra* by
155 molecular markers (Smulders et al. 2008b; Storme et al. 2001). Trees started to produce
156 pollen and seeds, ten to fifteen years after planting.

157 3.3 Plant material sampled

158 3.3.1 Poplar seedlings from the gravel banks

159 Poplar seedlings that naturally colonized the gravel banks of the natural floodplains of the
160 river Common Meuse were sampled on the banks along a 28 km-river section. We collected
161 leaf samples of 52 and 102 poplar seedlings in late summer of 2018 and 2019, respectively.
162 The geographical location of each sampled seedling was recorded. In 2018, poplar seedlings
163 were sampled randomly within the 28 km-section. In 2019, a more systematic survey was
164 performed within part of the same route, of about 9 km along the riverside; each gravel bank
165 was inspected by walking along the river. In case rejuvenation was present on a site, one to
166 twenty-tree seedlings were sampled across the rejuvenation site (mean: 1.6), depending on the
167 area of the colonized location. Only individuals that most likely originated from seed
168 germination and not from vegetative reproduction by root suckers were sampled. One young
169 leaf was collected from each seedling and dried in silica gel for DNA-analyses. The
170 estimated height of the majority of the sampled individuals was between 10 and 50 cm,
171 corresponding with the seedling or establishing stage (2-3 years) of riparian forest
172 development (Van Looy et al. 2005). The location along the river of the seedlings sampled is
173 given in Figure 1. The poplar seedlings were visually identified by their leaf morphology. One
174 young leaf was collected from each seedling and dried in silica gel for DNA-analyses.

175 3.3.2 Open pollinated progenies of *P. nigra*

176 *Populus* species are predominantly dioecious and thus obligatory outcrossers. Seeds of adult
177 open pollinated (OP) European black poplar females located in the study area were collected

178 in two successive years. In June 2018, seeds were collected at the location Groeskens from the
179 lower branches of the crown of six European black poplars. In June 2019, seeds were
180 collected on in total nine European black poplar females; six trees from location Groeskens
181 and three trees from location Negenoord. Seeds were collected from mature catkins, sown in
182 trays in the greenhouse (50% white peat / 50% black peat) within one week after collection.
183 For the seeds collected in 2019, germination percentage was determined. One young leaf
184 was collected from each seedling and dried in silica gel for DNA-analyses. The number of
185 seeds collected per open-pollinated *P. nigra* female is given in Table 1.

186 3.3.3 Reference material

187 In Europe, poplar species frequently used in breeding programmes in order to produce hybrids
188 are the European *P. nigra*, the North American cottonwoods *P. deltoides* W. Bartram ex
189 Marshall. and *P. trichocarpa* Torr. & A. Gray., and to a lower extent *P. maximowiczii* Henry
190 native to northeast Asia. The unit of cultivation and breeding in poplars is a clone, and
191 individual cultivars are normally represented by a single clone. We included 76 reference
192 samples with known taxonomic identity of frequently used pure poplar species and hybrids as
193 positive controls for verifying the species-specificity of the molecular markers. Furthermore,
194 reference samples of *P. nigra* and of frequently planted commercial male poplar cultivars
195 were included as potential fathers in the paternity-analyses. Reference material was obtained
196 from the clone collection of INBO located in Geraardsbergen, Belgium. The following taxa
197 were included: section Aigeiros with *P. deltoides* (4 samples), *P. nigra* (18 samples), *P. x*
198 *canadensis* (*P. deltoides* x *P. nigra*) (37 samples), section Tacamahaca with *P. trichocarpa* (4
199 samples), *P. trichocarpa* x *P. maximowiczii* (3 samples) and the intersectional hybrid; *P. x*
200 *generosa* (*P. trichocarpa* x *P. deltoides*) (10 samples). The list of reference material is given
201 in Supplementary Table S1.

202 3.4 Identification of hybrids

203 3.4.1 DNA extraction

204 Total genomic DNA was extracted from the sampled leaves with the QuickPick Plant DNA
205 kit (Bio-Nobile) using the MagRo 96-M robotic workstation (Bio-Nobile). For a subset of the
206 samples (10%), the integrity of the DNA was assessed on 1% agarose gels. DNA
207 quantification was performed with the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher
208 Scientific, Massachusetts, USA) using a Synergy HT plate reader (BioTek, Vermont, USA).

209 3.4.2 Chloroplast DNA marker

210 The chloroplast DNA (cpDNA) locus *trnDT* shows interspecific variation between *P. nigra*,
211 *P. deltoides* and *P. trichocarpa* (Heinze 1998; Meyer et al. 2018). Because of its maternal
212 inheritance and the female contribution of *P. deltoides* to the poplar clones frequently planted
213 in Europe, this marker offers a tool to identify the maternal contribution of *P. deltoides* / *P. x*
214 *canadensis*, *P. nigra* and *P. trichocarpa* in the poplar seedlings collected from the gravel
215 banks (e.g. Csencsics and Holderegger 2016; Heinze 1997; Ziegenhagen et al. 2008). We used
216 the conserved PCR primer pair *trnD/trnT* (Demesure et al. 1995) in combination with the
217 restriction enzyme *HinfI*. Genomic DNA (~20 – 50 ng) was used as template in the PCR
218 reactions. The PCR reaction conditions and restriction reactions were as described by Heinze
219 (1998). Reaction products and a 100 bp ladder were analysed by agarose gel electrophoresis.

220 3.4.3 Microsatellite analysis

221 We selected nine microsatellite loci that were found useful in former studies for the analysis
222 of natural hybridization events between cultivated poplars and their wild relative *P. nigra*
223 (Liesebach et al. 2010; Smulders et al. 2001; van der Schoot et al. 2000), and that were found
224 reliable for clonal fingerprinting in several species and hybrids of the sections Aigeiros and
225 Tacamahaca (Dayanandan et al. 1998; Liesebach et al. 2010; Rahman et al. 2000). All
226 markers are unlinked (Cervera et al. 2001; Gaudet et al. 2008). According to former studies,
227 five loci produce species-diagnostic alleles for *P. deltoides* and/or *P. trichocarpa*: PMGC14
228 (Fossati et al. 2003; Liesebach et al. 2010), PMGC456 (Liesebach et al. 2010), PMGC2163
229 (Liesebach et al. 2010), WPMS09 (Fossati et al. 2003; Liesebach et al. 2010) and WPMS20
230 (Liesebach et al. 2010). SSR analysis was performed on the OP progenies, the seedlings from
231 the gravel banks and the reference samples as described by Smulders et al. (2001) and van der
232 Schoot et al. (2000). Ten samples were replicated twice starting from a second DNA-extract
233 to calculate the genotyping error rate. PCR products were run on an ABI3500 Genetic
234 Analyzer (Applied Biosystems) and genotypes were analysed with the GeneMapper v.6.0
235 software package. Samples with fewer than 8 scored loci were discarded from the data
236 analysis. Details on the microsatellite loci are given in Table 2.

237 3.4.4 Data analysis

238 The maternal origin of the poplar seedlings sampled on the gravel banks was investigated
239 using the cpDNA locus *trnDT*. The poplar seedlings from the gravel banks and the OP
240 progenies were analysed for the presence of diagnostic alleles of *P. deltoides* and *P.*
241 *trichocarpa* on the five diagnostic SSR loci. A diagnostic allele for *P. deltoides* in *P. nigra*
242 maternal offspring indicates a backcrossed individual with *P. x canadensis* paternity as a
243 result of a natural hybridization event between a cultivar of *P. x canadensis* and a native *P.*
244 *nigra*.

245 We also performed a paternity analysis on the OP progenies using the microsatellite data to
246 determine pollination events of frequently planted male poplar cultivars included in the
247 reference samples. We used a likelihood-based approach implemented in the program
248 CERVUS 3.0.7 (Marshall et al. 1998) with the reference samples described above (48;
249 females excluded) as potential fathers, given a known mother. The paternity assignment
250 technique used by CERVUS requires the knowledge of the total number of candidate males in
251 the population and the proportion of the candidate males sampled for the simulation-based
252 approach to assess the confidence of the assignments. For the simulation, we used the LOD
253 distribution and we assumed 50 candidate fathers and a proportion of 0.3 candidate fathers
254 sampled, allowing an error rate of 0.01 and allowing a mismatch between parent and offspring
255 on one locus taking into account somatic mutations and null alleles.

256 4. Results

257 4.1 Diagnostic microsatellite alleles

258 The percentage missing data for the total microsatellite data was 0.07%. Replicated samples
259 resulted in identical multilocus genotypes. For the seedlings sampled from the gravel banks,
260 two multilocus genotypes were shared by two and three sampled seedlings, respectively. It is
261 possible that some individuals were sampled twice, in 2018 and 2019. Only one genotype
262 among these replicates was kept in the final dataset. The microsatellite data confirm the

263 presence of species-diagnostic alleles reported in former studies by Liesebach et al. (2010)
264 and Fossati et al. (2003) for PMGC14, PMGC456, PMGC2163 and WPMS09 but not for
265 WPMS20. For PMGC14, the alleles 192 and 198 were typical for all reference clones of *P.*
266 *deltoides* and for *P. x canadensis*, and the allele 200 was typical for all reference clones of *P.*
267 *trichocarpa*. For WPMS09, allele 232 was present in the reference clones of *P. deltoides* and
268 of *P. x canadensis*, except for 16 *P. x canadensis* reference clones that were homozygote for
269 this locus. This can be explained by their genetic origin (i.e. F2 or back-cross clones) or by
270 the presence of a null allele. For WPMS20, the characteristic allele for *P. deltoides* (allele
271 200) also frequently occurred in *P. nigra*, which was also reported by Liesebach et al. (2010).
272 Furthermore, species-specific alleles were present at locus PMGC2163 (*P. deltoides*: allele
273 185, *P. trichocarpa*: alleles 199, 200) and locus PMGC456 (*P. deltoides*: allele 82, *P.*
274 *trichocarpa*: alleles 93, 109, *P. nigra*: allele 75).

275 4.2 Poplar seedlings from the gravel banks

276 From the 154 seedlings sampled on the gravel banks, 135 showed a clear banding pattern for
277 the cpDNA locus *trnDT*. From the latter, 133 (98.5%) poplar seedlings showed a *P. nigra*-
278 specific *trnDT* marker-fragment (~880 bp). These seedlings also showed a *P. nigra*-like leaf
279 morphology. The typical gene variant of *P. nigra* observed on the maternal inherited
280 diagnostic cpDNA locus *trnDT* indicated that they originated from a female *P. nigra*.
281 Furthermore, two (1.5%) seedlings showed a *P. trichocarpa*-specific cpDNA fragment
282 (~1000bp). These two samples with a *P. trichocarpa*-like leave morphology were collected on
283 an upstream distance of 6 km from the nearest *P. nigra*. No seedlings were detected with a
284 fragment length specific of *P. deltoides* (~1135bp). The results of the diagnostic microsatellite
285 alleles confirmed the presence of species-specific *P. trichocarpa* alleles at loci PMGC147,
286 PMGC2163 and PMGC456 for the two seedlings with a *P. trichocarpa*-specific *trnDT*
287 marker-fragment. For one of these two seedlings, species-specific alleles of *P. trichocarpa*
288 were homozygous, suggesting that this seedling could have originated from two *P.*
289 *trichocarpa* or *P. x generosa* cultivars. Furthermore, two more seedlings sampled in the field
290 showed species-specific alleles of *P. deltoides* in heterozygous state at three and four
291 diagnostic loci, respectively. Given their *P. nigra*-specific *trnDT* marker-fragment, they likely
292 originated from a *P. nigra* x *P. x canadensis* back-cross. Summarised, four (2%) out of the
293 154 young poplars analysed from the gravel banks exhibited genes of exotic, cultivated poplar
294 species.

295 4.3 Identification of hybrids in open pollinated progenies

296 Viable seedlings were obtained from six and five black poplar trees in 2018 and 2019,
297 respectively. The mother trees presented only two different genets. The mean germination
298 percentage for the 1492 seeds sown in 2019 was low (2.9% ; range of 0% to 6.6%). In total,
299 we obtained 74 viable seedlings of which 72 were successfully genotyped (Table 1). We
300 detected no alleles species-specific to *P. deltoides* or *P. trichocarpa* in the 72 analysed
301 seedlings from the OP *P. nigra* progenies, suggesting the absence of backcrossed individuals
302 with *P. x canadensis* or *P. x generosa* as potential fathers.

303 However, the paternity analysis revealed *P. x canadensis* a highly likely father for three
304 seedlings from the OP progeny from seed harvested in 2018 on one mother tree (ID 2.16) at
305 location Groeskens. Paternity was determined with 95% confidence for 27 (38%) seedlings.

306 When using a relaxed confidence level of 80%, paternity was assigned to 48 (67%) seedlings.
307 For 24 (33%) seedlings, the paternity remained unresolved. For one seedling paternity was
308 assigned to *P. x canadensis* cv. Serotina de Champagne using a relaxed confidence level and
309 with no paternity-offspring mismatches. Also for a second seedling from the same mother
310 tree, *P. x canadensis* cv. Serotina de Champagne was the most likely father with a mismatch
311 for only one locus (PMGC14), possibly due to a somatic mutation. A third seedling from the
312 same OP progeny showed *P. x canadensis* cv. Serotina as the most likely father, although
313 mismatch was observed at two loci (PMGC14, PTR07). The other determined paternities
314 were assigned to trees of *P. nigra*. No offspring was assigned to the male cultivar *P. nigra* cv.
315 Italica, nor to the nearby planted male *P. x canadensis* cv. Robusta or to the reference
316 cultivars of *P. x generosa*.

317

318 5. Discussion

319 The results of this study illustrate the importance of in situ reinforcement of black poplar to
320 reduce the potential impact of exotic poplar cultivars in European floodplains in case natural
321 river dynamic processes are restored and adult black poplar trees are scarce. The numbers of
322 young poplar seedlings that colonized the gravel banks of the river Meuse exceeded 100 fold
323 the initial number of seedlings observed by Vanden Broeck et al. (2004). Additionally the
324 number of seeds and seedlings with exotic poplar genes were diminished to a very low
325 proportion compared to the situation before the restoration.

326 5.1 Limited interspecific matings

327 The majority (150, 98%) of the poplar seedlings from the gravel banks analyzed were
328 identified as 'pure' *P. nigra* seedlings; the typical gene variant of *P. nigra* observed in the
329 maternal inherited diagnostic cpDNA locus *trnDT* indicated that they originated from a
330 female *P. nigra*. They could not originate from cultivated first-generation (F1) *P. x*
331 *canadensis* hybrids, as cultivated clones of *P. x canadensis* are the result from artificial
332 crosses between a female *P. deltoides* and a male *P. nigra*, thus carrying the *P. deltoides*
333 haplotype (Heinze 1998; Zsuffa 1974). Furthermore, the absence of microsatellite alleles of *P.*
334 *deltoides* or *P. trichocarpa* suggests that they were fathered by *P. nigra*. Only a small number
335 (four; 2%) of young poplars analyzed from the gravel banks exhibited genes of exotic,
336 cultivated poplars. Of the latter, two seedlings showed *P. trichocarpa* genes while the other
337 two resulted from a first-generation back-cross (BC1) of a male *P. x canadensis* and a female
338 *P. nigra*. No gene variants of exotic poplar species were detected in the OP progenies. This
339 was largely confirmed by the paternity analyses, except for three seedlings (4%) of which two
340 were likely fathered by *P. x canadensis* cv. Serotina de Champagne and one seedling by *P. x*
341 *canadensis* cv. Serotina. It must be noted that these two cultivars are clearly different and not
342 related, although their names suggest otherwise. They show different alleles at seven out of
343 the nine microsatellite loci genotyped and also show differences in leaf morphology as was
344 noted by Broekhuizen (1960). No paternity was assigned to the male cultivar *P. nigra* cv.
345 Italica (Lombardy poplar), one of the most planted poplar cultivars. In Belgium, the
346 Lombardy poplar generally flowers before the majority of the native black poplars (Vanden
347 Broeck et al. 2003a), which may not be the case in more Southern regions (Chenault et al.
348 2011). Absence of flowering synchrony may explain why no paternity was assigned to this
349 cultivar or to the nearby planted male *P. x canadensis* cv. Robusta in this study.

350 To our knowledge, this is the first study combining diagnostic chloroplast and nuclear
351 molecular markers with a paternity analysis to detect interspecific hybridization events
352 between native black poplar and poplar cultivars. The probability to detect gene variants of *P.*
353 *deltoides* using four diagnostic nuclear microsatellite loci and according to Mendelian rules in
354 first-backcross generations (BC1) is 93.75%, but decreases to 68.36% for second back-cross
355 generations (BC2) (see Bialozyt et al. 2012). This could explain why gene variants of *P.*
356 *deltoides* remained undetected in three seedlings from the OP progenies to which a cultivar of
357 *P. x canadensis* was assigned as the most likely father. In this study, the paternity analysis
358 increased the reliability to identify further generation back-cross events in the OP progenies.
359 However, the power of a paternity analysis depends on the potential male parents included in
360 the analysis and the assumptions made on the proportion of potential fathers sampled. It is

361 possible that some second back-cross events remained undetected because of these sampling
362 limitations.

363 5.2 Pollen and seed pools affecting exotic gene flow

364 The contrast in the number of interspecific mating events before and after the river and
365 vegetation restoration activities is remarkable. In 1999 - 2001, the majority of the seedlings
366 sampled on the gravel banks (27/29; 93%) showed a *P. trichocarpa* fragment at the cpDNA
367 locus *trnDT* and/or species-specific alleles of *P. deltoides* (Vanden Broeck et al. 2004). In
368 contrast to this study, gene variants of exotic poplar species were also detected in the majority
369 of the seedlings (32/34; 94%) of the OP progenies of an isolated female *P. nigra* in the study
370 area in 1999 - 2001 (Vanden Broeck et al. 2004). At that time, *P. nigra* was extremely rare in
371 the study area as a result of habitat reduction and fragmentation; the native species was only
372 represented by a few old trees, in contrast with the widely planted poplar cultivars (Vanden
373 Broeck et al. 2004). The strong decrease in population size combined with the habitat
374 fragmentation of the native *P. nigra* increased the opportunities for contact with cultivated
375 poplar clones and provided at that time excellent opportunities for the production of hybrid
376 seeds and seedlings. Poplars are prolific seed producers; one tree can produce over 50 million
377 of seeds in a single season (Oecd 2000). Nevertheless, poplar hybrids are generally
378 characterized by reduced fertility relative to parental species, with significant lower pollen
379 production and seed viability in F1-hybrids (Stettler et al. 1996). It is therefore likely that the
380 seed production and seed viability of *P. nigra* outperformed those of the cultivated poplars.
381 Also, pollen load composition and size could have been an important process in limiting the
382 production of hybrid progeny. The low frequencies of interspecific mating relative to
383 intraspecific mating using a pollen mixture of different taxa in controlled crosses suggest a
384 greater competitive ability for conspecific pollen (Benetka et al. 2002; Gaget et al. 1989;
385 Rajora 1989; Vanden Broeck et al. 2003b). As a result, the relative fertilization success of a
386 pollen tree depends upon the taxon constitution of the pollen mix and thus on the number and
387 taxon of the producing pollen trees in the study area.

388 In this study, the few seedlings showing exotic poplar genes were located at distances of > 1
389 km from the nearest reintroduction sites of black poplar. This indicates that hybrid poplars
390 still reproduce in the study area, in particular on sites located further away from the
391 reforestation sites of black poplar. Establishing more seed sources of black poplar over the
392 river stretch could further reduce hybrid reproductive success along the Common Meuse.
393 Although conspecific siring of female *P. x canadensis* is enhanced by the presence of pollen
394 of black poplar (Vanden Broeck et al. 2012), the higher local density of male and female
395 black poplars appears to guarantee the establishment of mainly pure black poplar seedlings
396 nearby the parental stands. Considering the possibility of long distance gene flow (Imbert and
397 Lefèvre 2003), investigations of interspecific mating events would ideally cover the whole
398 river stretch where natural habitat is restored.

399 5.3 Conservation implications

400 The results of this study illustrate the importance of in situ reinforcement of black poplar
401 populations to reduce the potential impact of exotic poplar cultivars along European
402 floodplains where natural river dynamic processes are restored and adult black poplar trees
403 are scarce. The potential risks for gene introgression from exotic poplars into black poplar is

404 indeed highly variable between geographic locations and river sites, largely depending on
405 habitat fragmentation, flowering synchrony and the composition of the pollen and seed pool at
406 the specific site. While interspecific gene flow seems to be absent in large natural populations
407 of black poplar, like in France along the rivers Loire, Garonne and Drôme (Imbert and
408 Lefèvre 2003), significant gene flow from exotic poplars in riparian floodplains is reported
409 along the rivers Rhine and the Elbe in Germany (Meyer et al. 2018; Ziegenhagen et al. 2008),
410 the Morava River in the Czech Republic {Pospiskova, 2006 #832}, the Ticino river in Italy
411 (Fossati et al. 2003), the Rhine river in The Netherlands (Arens et al. 1998; Smulders et al.
412 2008a) and the rivers Thur and Reuss in Switzerland (Csencsics and Holderegger 2016). This
413 case study along the Common Meuse, demonstrates that reintroduction and reinforcement of
414 native black poplar populations can reverse the situation from high to low risk of
415 introgression with cultivated poplars on a relatively short time scale. By changing the
416 composition of the seed and pollen pool, black poplar nowadays has a competitive advantage
417 compared to the exotic poplars in colonizing the restored river banks along the Common
418 Meuse.

419 Hundreds of young black poplar seedlings colonized the gravel banks. With an estimated age
420 for the majority of the seedlings of two to three years old, they survived the germination stage
421 and currently present the establishment stage of softwood forest development with the
422 development of dense thickets (Van Looy 2006). The survival of poplar seedlings depends on
423 the combination of many factors broadly related to the availability of water and oxygen
424 (Hughes et al. 2001). As a result of the critical conditions required by seedlings, successful
425 establishment only occurs in some years, and within well-defined elevational bands (Hughes
426 et al. 2001). Furthermore, established seedlings at lower river levels are frequently subject to
427 later removal or damage by drought, flooding, sediment burial or mechanical disturbance
428 (Hughes et al. 2001; Jacquemyn et al. 2006). Seedling survival should therefore be monitored
429 over larger time scales to evaluate the success of this restoration project and to predict riparian
430 forest development along the river Common Meuse. Management and conservation plans
431 should include further monitoring of the seedling colonization and survival, the effective
432 population size and the introgression of foreign genes in black poplar across multiple
433 generations. Also, the vegetative spread of cultivated hybrids, which was not the focus of this
434 study, should be taken into consideration in predicting the development of riparian forest
435 ecosystems. The data can then be used for the construction of habitat models to identify
436 occupied or potential habitat and to predict riparian forest development in the restored
437 floodplain area (Debeljak et al. 2015; Van Looy et al. 2005).

438 **6. Conclusions**

439 Almost two decades after the reintroduction of black poplar along the river Common Meuse,
440 the constitution of the seed and pollen pool changed in the study area thereby benefitting the
441 reproduction of the native species at the expense of the reproduction capacity of the exotic
442 poplar species. Increasing the density of male and female black poplars appears to guarantee
443 the establishment of mainly pure black poplar seedlings nearby the parental stands.
444 Nevertheless, exotic poplar species still reproduce at larger distances from the revegetation
445 sites and colonize restored habitat thereby competing for resources with the native species.
446 Therefore, increasing the number of native black poplar seed sources along the river stretch
447 will help the native black poplar to maintain its competitive advantage over the exotic poplars

448 and will contribute considerably to the success of riparian forest development along the
449 Common Meuse.

450 **7. Author statements**

451 AVdB, KVL: conceptualization; AVdB: methodology, formal analysis, writing - original
452 draft; KC, AVB, KVL: writing - review & editing; SN, NDR: methodology, resources. All
453 authors have read and approved the final version.

454 **8. Declaration of competing interest**

455 The authors declare that they have no potential conflicts of interest.

456

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464 interpretation of data, or in the decision to submit the article for publication.

465 **11. Data profile**

466 Data will be made available after acceptance of the manuscript for publication by the data
467 repository DRYAD.

468

469 **12. References**

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679

680 **13. Tables**

681 **Table 1.** The number of seeds collected, seed germination percentage and the number of
682 seedlings analyzed per open pollinated *Populus nigra* female.

| Collection year | Location | P. nigra tree ID | # seeds | seed germination (%) | # seedlings analysed |
|-----------------|-----------|------------------|---------|----------------------|----------------------|
| 2018 | Groeskens | 2.16 | NA | NA | 28 |
| 2018 | Groeskens | 5.20 | NA | NA | 1 |
| 2019 | Negenoord | 1 | 160 | 2.50 | 4 |
| 2019 | Negenoord | 2 | 300 | 3.00 | 9 |
| 2019 | Negenoord | 3 | 300 | 5.67 | 17 |
| 2019 | Groeskens | 5.25 | 300 | 3.00 | 8 |
| 2019 | Groeskens | 5.20 | 76 | 6.58 | 5 |
| Total | | | | | 72 |

683

684

685 **Table 2** Microsatellite loci used for taxonomic classification and paternity analysis.

| | Locus | Linkage group | Repeat motif | Fragment size (bp) | Annealing Temperature (°C) | Reference |
|---|-----------|---------------|--------------|--------------------|----------------------------|------------------------------|
| 1 | PMGC_14 | XIII | CTT | 179 - 227 | 52 | PMGC |
| 2 | WPMS_05 | XII | GT | 263 - 291 | 52 | van der Schoot et al. (2000) |
| 3 | PMGC_2163 | X | GA | 198 - 220 | 52 | PMGC |
| 4 | PMGC_456 | II | GA | 80 - 136 | 52 | PMGC |
| 5 | WPMS_16 | VII | GTC | 128-167 | 52 | Smulders et al. (2001) |
| 6 | WPMS_09 | VI | GT | 244 -294 | 57 | Smulders et al. (2001) |
| 7 | PTR2 | IX | TGG | 207 -228 | 57 | Dayanandan et al. (1998) |
| 8 | WPMS_20 | XIII | TTCTGG | 224 - 242 | 57 | Smulders et al. (2001) |
| 9 | PTR7 | XII | (CT)5AT(CT) | 230 -250 | 57 | Rahman et al. (2002) |

686

687

688 **14. Figure**

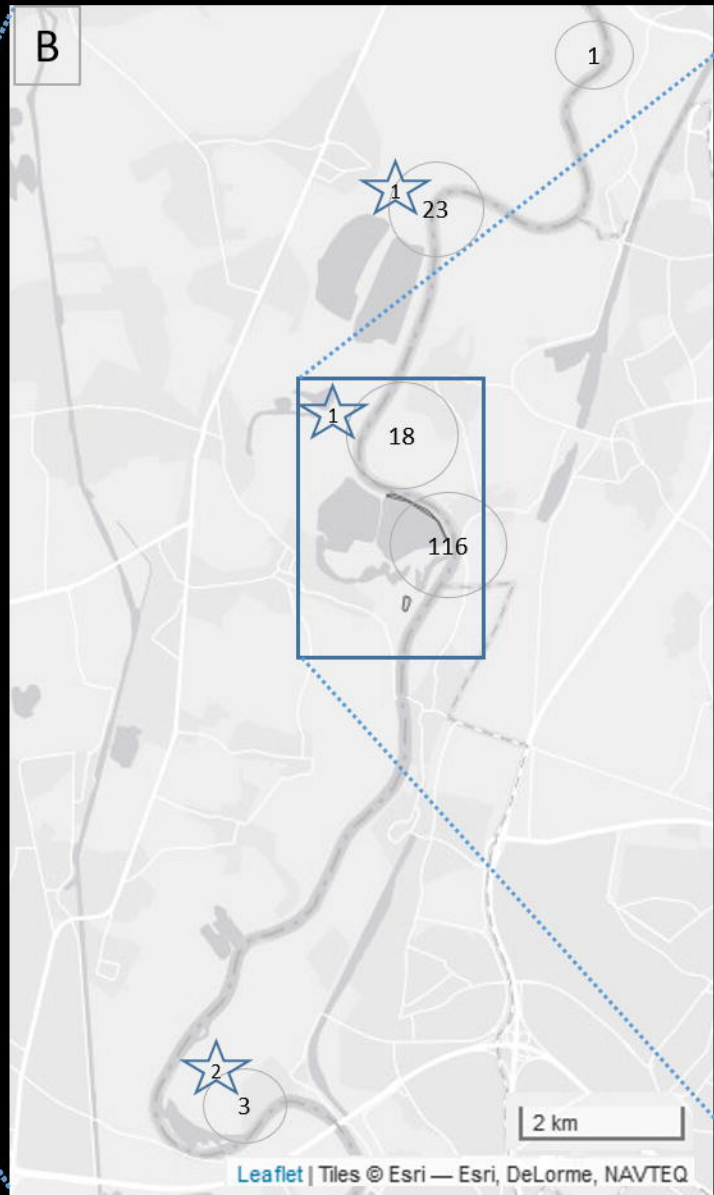
689 **Figure 1. A.** Location of the river Common Meuse. **B.** Map of the study site and sampling
690 locations. Nearby sampled poplar seedlings are clustered and the total number of clustered
691 samples is given in the circles. Stars indicate the position of hybrid poplar seedlings sampled.
692 **C.** Location of the introduced *Populus nigra*.

693



694 **15. Supplementary material**

695 [Table S1](#). List of *Populus* species and poplar cultivars with known taxonomic identity used as
696 reference samples. References to the cultivars were obtained from the International Register
697 of Poplar Cultivars (IPC – FAO)

698



Legend

-  Number of poplar seedlings sampled
-  Hybrid poplar seedling sampled