Individual small in-stream barriers contribute little to strong local population genetic structure five strictly aquatic macroinvertebrate taxa

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Abstract

Water flow in river networks is frequently regulated by man-made in-stream barriers. These obstacles can hinder dispersal of aquatic organisms and isolate populations leading to the loss of genetic diversity. Although millions of small in-stream barriers exist worldwide, such as weirs below 2 m or tunnels and pipes, their impact on the dispersal of macroinvertebrates with strictly aquatic life cycles is unclear. We therefore assessed the effects of such barriers on the population structure and effective dispersal of five macroinvertebrate species with strictly aquatic life cycles: the amphipod crustacean *Gammarus fossarum* (clade 11), three snail species of the *Ancylus fluviatilis* species complex and the flatworm *Dugesia gonocephala*. We studied populations at nine weirs and eight culverts (3 pipes, 5 tunnels), built 33-109 years ago, in the heavily fragmented catchment of the river Ruhr (Sauerland, Germany). To assess fragmentation and barrier effects, we generated genome-wide SNP data using ddRAD sequencing and evaluated clustering, differentiation between populations up- and downstream of each barrier and effective migration rates among sites and across barriers. Additionally, we applied population genomic simulations to assess expected differentiation patterns under different gene flow scenarios. Our data show that populations of all species are highly isolated at regional and local scales within few kilometres. While the regional population structure likely results from historical processes, the strong local differentiation suggests that contemporary dispersal barriers exist. However, we identified significant barrier effects only for pipes (for *A. fluviatilis* II and III) and few larger weirs (>1.3 m; for *D. gonocephala*). Therefore our data suggest that most small in-stream barriers can probably be overcome by all studied taxa frequently enough to prevent fragmentation. Thus, the barriers leading to population isolation after few kilometres still need to be identified. Specifically, it remains to be tested if the strong local differentiation is a result of a cumulative effect of small barriers, or if e.g. larger in-stream barriers, land use, chemical pollution, urbanisation, or a combination of these factors impede gene flow.

Introduction

Rivers in the Anthropocene are in crisis due to various human activities (Jackson et al., 2001; Vörösmarty et al., 2010). In particular, natural water flow has been altered substantially over the past few centuries by the construction of in-stream barriers, such as dams, weirs (Grill et al., 2015; Nilsson et al., 2005; Zarfl et al., 2015) and culverts (David et al., 2014; Torterotot et al., 2014; Wheeler et al., 2005). Such human-induced habitat fragmentation is considered a major threat to biodiversity (Hudman & Gido, 2013). In isolated populations, the effects of genetic drift and inbreeding are higher, thereby increasing the risk of genetic diversity loss (e.g., Vrijenhoek, 1998) and extinction (Bijlsma & Loeschcke, 2012). To predict population resilience and long-term adaptability, it is therefore crucial to understand species-specific dispersal patterns (Hughes et al., 2008) and to identify actual barriers to dispersal.

Even within species, dispersal rates can differ between areas and sites as site-specific environmental factors such as water chemistry, land use and urbanisation can influence effective dispersal rates making predictions difficult. Another important factor that influences population genetic structure are in-stream barriers. Impacts of such barriers have been shown mainly for populations of different fish species (e.g., Hansen et al., 2014; Horreo et al., 2011; Junker et al., 2012; Torterotot et al., 2014; Van Leeuwen et al., 2018). In contrast, for freshwater invertebrate taxa, the effect of in-stream barriers is often unknown. Although barrier effects have been reported (David et al., 2014; Dillon, 1988; Resh, 2005), they are highly species-specific (Tonkin et al., 2014) owing to divergence in life history traits. While more studies have focused on large barriers such as dams or reservoirs, little is known about the effects of smaller obstacles such as weirs and culverts that can occur at high densities, with less than 1 km between barriers (Belletti et al., 2020; Dumont et al., 2005). Only limited information about the properties of these small barriers exists, especially with respect to barrier age, shape and impoundment areas, making it difficult to predict their impact on genetic structure. Further, in-stream barriers might not act as complete barriers, i.e. they may not prevent gene flow equally in both directions, but only, or stronger, in upstream direction. This asymmetry makes it more difficult to detect effects. When focusing on macroinvertebrates, other confounding factors that limit the detectability of barrier effects are the

often large populations sizes (limiting genetic drift effects) and low dispersal abilities (e.g., in strictly aquatic taxa). Low effective dispersal alone can generate a strong genetic background structure, which needs to be accounted for when studying impacts of in-stream barriers on genetic structure (Coleman et al., 2018). Therefore, beside studying the direct effect of barriers, it is also important to know the general population structure of the species in the area to be able to interpret the results. In addition to temporal sampling or space-for-time study designs to distinguish between background genetic structure and barrier effects (Coleman et al., 2018), population genetic simulations can improve the interpretation of empirical data by generating null models under specific assumptions, for example life history traits (Hoban et al., 2012). Considering the relatively young age of many man-made barriers (typically tens of years to a few hundred years) and the often large population sizes of macroinvertebrate species, high density genomic markers, like single nucleotide polymorphisms (SNPs), are needed to resolve fine-scale patterns of genetic structure (Whiterod et al., 2017).

In this study, we first performed comparative population genomic analyses to assess effective dispersal of different and locally abundant stream macroinvertebrate taxa in a heavily fragmented German low mountain range stream network. The second aim was then to evaluate the impact of small in-stream barriers on population genetic structure in these species. To achieve these aims, we generated genome-wide SNP data by double digest restriction site-associated sequencing (ddRAD-seq, Peterson et al., 2012). To account for species- and site-specific genetic background structure, reference sites were sampled in each stream. Further, the distances between sites were chosen within the dispersal ability of all species (~200 m), so that without a barrier, gene flow should be high. All selected species, i.e. the amphipod crustacean *Gammarus fossarum* clade 11 (after Weiss et al., 2014), the flatworm *Dugesia gonocephala* and three snail species of the *Ancylus fluviatilis* species complex (*A. fluviatilis* I, II, III; Weiss et al., 2018), are strictly confined to the aquatic environment (i.e., are hololimnic) and commonly occur in small European streams. They differ in feeding type, reproductive strategy and dispersal mode. Based on specific information on ecology, life history traits (obtained from Baršiene et al., 1996; Brittain & Eikeland, 1988; Burch, 1962; de Vries, 1986; Eder et al., 1995; Elliott, 2003; MacNeil et al., 1997; Minshall & Winger, 1968; Moog, 1995; Nesemann & Reischütz, 1995; Patrick et al., 2014; Schmidt-

Kloiber & Hering, 2015) and initial genetic data for *G. fossarum* (Alp et al., 2012; Weiss & Leese, 2016), we derived predictions concerning the strength of in-stream barrier effects and population structure (Figure 1). In order to put results into context, we simulated population genomic data as null models to test how species-specific life history traits influence the detectability of barrier effects. Based on mobility and population size (Figure 1), we hypothesised that *G. fossarum* shows the lowest and *A. fluviatilis* I, II and III the strongest population structure, while it is intermediate for *D. gonocephala*. Further, concerning barrier effects, we hypothesised that barriers increase genetic differentiation compared to control sites and that weirs have stronger effects than culverts. Additionally, we hypothesised that the effect of weirs depends on barrier properties, with the highest, steepest and smoothest barriers having the strongest effect. For culverts, we hypothesised that pipes have stronger effects than tunnels and that the effect depends on the length, diameter and bottom substrate of the culvert, with longer culverts with a smaller diameter and concrete bottom (instead of natural sediment) having the strongest effect. Furthermore, we hypothesise that effects of barriers on differentiation is species-specific, with strongest effects expected for *A. fluviatilis* I, II and III and weakest for *G. fossarum.*

Materials and Methods

Study design and study sites

The study region was the river Ruhr catchment (North Rhine-Westphalia, Sauerland, Germany), with many weirs and culverts, often separated by less than 1 km (Dumont et al., 2005). Taxa sampled were *G. fossarum* clade 11*, D. gonocephala* and *A. fluviatilis.* The three species *A. fluviatilis* I, II and III can only be identified by ddRAD analyses (Weiss et al., 2018); accordingly, specimens were sampled and processed in the lab as a single species. The sampling scheme was designed to systematically test the impact of two types of in-stream barriers, weirs (abbreviated QB [for German 'Querbauwerk']) and culverts (VR [for German 'Verrohrung']), on gene flow. To compare gene flow among populations with and without a barrier in the same stream, specimens were sampled from at least three sampling sites (S), so that two sites were sampled upstream (S1 and S2) or downstream (S3 and S4) of the barrier with no

barrier in-between. Where possible, reference sites were sampled up- and downstream of the barrier. In one case (VR17), three reference sites were sampled upstream of the barrier. If tributaries joined the stream within the sampling range, specimens were collected at these locations (abbreviated N). In two cases, the scheme was extended to contain five sites (S0–S4) with a barrier downstream of S1 and S2 (VR6 and QB27). Based on the dispersal range of all target taxa, 200 m was chosen as the distance between sampling sites, including sites separated by a barrier. The sampling stretch containing all individual sampling sites associated with one barrier is in the following called 'barrier site' (see Figure 2 and 3). Specimens were collected at 15 barrier sites consisting of 17 barriers (Figure 2), including eight culverts (8–120 m in length) and nine weirs (0.6–1.65 m in height) (Table 1, Figure S1 for exemplary pictures). At individual sampling sites, 5–12 specimens per species were sampled, but not all target species occurred at all sites (see Table S1 for details).

Genotyping

DNA was extracted from muscle tissue using a salt-extraction protocol as described in Weiss & Leese (2016) for 344 *D. gonocephala* and 123 *A. fluviatilis* specimens. For *G. fossarum*, DNA from 315 specimens from a previous study (Weiss & Leese, 2016) was used. Four ddRAD libraries were generated for *D. gonocephala* (containing 83, 83, 87 and 89 samples), four for *G. fossarum* (92, 66, 65 and 95 (3 repeated from library 1)) and two for *A. fluviatilis* (62 and 61). For *A. fluviatilis*, data from three previously generated ddRAD libraries (269 specimens, Weiss et al., 2018) were also used, resulting in 389 specimens. The ddRAD libraries were generated according to the protocol described in Vendrami et al. (2017), with modifications described in Weiss et al. (2018) and Weigand et al. (2018). An overview of the protocol with species-specific details is given in Appendix S1 and sample preparation details are given in Table S2. All samples for each library were pooled with equimolar concentrations and sequenced on an Illumina HiSeq 2500 sequencer to obtain 125 bp paired-end reads (Eurofins Genomics Europe Sequencing GmbH; Constance, Germany).

153 **ddRAD data analysis**

154 For a better overview, all important analysis steps are summarised in the workflow in Figure 3. Pre-
155 processing of ddRAD data for each sequencing library was conducted as described in Weiss et al. (2018).
156 After pre-processing, denovo_map.pl of Stacks v.1.34 (Catchen et al., 2013) was used to identify loci
157 and genotypes. Stacks was run with eight different settings per species to identify the optimal parameter
158 settings (Paris, Stevens, & Catchen, 2017, see Appendix S1 for details). Stacks results were exported
159 from stacks databases with export_sql.pl (minimum stack depth 8). For *D. gonocephala,* aneuploid
160 cytotypes are known besides diploids, with chromosome numbers similar to triploids (de Vries, 1986).
161 Therefore, individual ploidy levels based on the expected allelic coverage was estimated using the R-
162 script ploidyCounter.R (Rozenberg, https://github.com/evoeco/radtools/, see Weigand et al. 2018 for
163 details). However, this ploidy estimation is indirect and aneuploids with high chromosome numbers
164 cannot be distinguished from real triploids. Therefore, specimens with coverage distribution plots as
165 expected for triploids are called 'potential triploids' hereafter. Direct estimation of ploidy levels, e.g.
166 via flow cytometry was not possible from the ethanol fixed material. All analyses were also conducted
167 excluding potential triploids, but as they produced similar results only analyses including all specimens
168 are presented here.

169 Further analyses were performed using Snakemake workflows (Köster & Rahmann, 2012),
170 combining stacks2fasta.pl (Macher et al., 2015) and several in-house python and R-Scripts for data
171 reformatting, filtering and population genetic analyses. Parameter settings were similar for each species,
172 except for *Ancylus* species, where parameters were adjusted when possible to account for polyploid
173 genomes.

174 First, Stacks parameter and locus filtering settings were optimised, as described in detail in
175 Appendix S1. General population structure was analysed with individuals divided into populations
176 according to barrier sites, i.e. pooling specimens from individual sampling sites at a barrier site to one
177 population (Figure 3, step 6). The following locus filtering settings were chosen: 1–12 SNPs/locus (only
178 one used), minor allele frequency 1%, locus present in at least 80% of individuals per population and
179 95% of all individuals. Further, specimens with too few reads, or more than 20% missing data were

excluded. Basic population genetic statistics, e.g. observed heterozygosity (H_O), observed gene diversity (H_S), overall gene diversity (H_T) and overall F_{ST} and F_{IS} were calculated using the R-package hierfstat (Goudet, 2005) in R v. 3.3.2 (R Core Team, 2015). Further, principal component analyses (PCAs; Patterson et al., 2006) were performed and individual ancestry coefficients were estimated based on sparse nonnegative matrix factorisation algorithms (sNMF; Frichot et al., 2014) using the R-package LEA (Frichot & François, 2015) to identify genetic clusters. Both methods are suitable for polyploid and mixed ploidy data as they do not assume Hardy–Weinberg equilibrium (Dufresne et al., 2014; Frichot et al., 2014). For sNMF analyses, number of clusters tested were k = 1–15 for *Ancylus* species and k = 1–17 for *G. fossarum* and *D. gonocephala*, and analyses were run with 30 replicates and 100,000 iterations per replicate. In addition, Neighbour-Net networks (Bryant & Moulton, 2004) were calculated using SplitsTree v. 4.14.5 (Huson & Bryant, 2006). Pairwise F_{ST} values (after Weir & Cockerham, 1984) between barrier sites were calculated and significance was tested by bootstrapping over loci (10,000 replicates; 0.025/0.975 confidence intervals) using the R-package hierfstat. H_O and allelic richness (AR) in each population were calculated using the R-package diveRsity (Keenan et al., 2013). Further, the divMigrate function (Sundqvist et al., 2016) of this R-package was used to assess directional relative migration rates and to detect asymmetries in gene flow using Nei's G_{ST} (Nei, 1973). To evaluate correlation of genetic and geographic distances (isolation by distance, IBD), Mantel tests were conducted using the R-package vegan (Oksanen et al., 2019). As a measure of genetic distance, pairwise F_{ST} values were calculated between all individual sampling sites (S). For geographic distances, either straight-line or waterway distances were used. Both distances were calculated using QGIS v. 2.14.14 (http://qgis.org) with the same stream map used for the visualisation of sampling sites and population structure, provided by the federal state authority LANUV (Gewässerstationierungskarte des Landes NRW © LANUV NRW (2013)).

Finally, the effects of different in-stream barriers on gene flow among populations up- and downstream of barriers were analysed (Figure 3, step 7). To enable detection of low differentiation among adjacent sites, loci were exported separately for each barrier site with the same filter settings used for previous analyses, except the minor allele frequency was set to 5% and loci had to be present

207 in 90% of specimens to account for the smaller sample sizes. For barrier analyses, two population
208 definitions were used. First, all analyses were conducted with barrier sites as individual populations, i.e.
209 specimens from individual sampling sites up- and downstream of a barrier were pooled (S upstream/S
210 downstream). Second, all individual sampling sites (S) were treated as different populations (see Figure
211 3). Basic population genetic statistics, sNMF (k = 1-6), H_O and AR per population and pairwise F_{ST} were
212 calculated as before. The number of private alleles in each population was calculated using an in-house
213 python script. To account for differences in sample size, larger populations were randomly subsampled
214 30 times to match the smallest population size and the mean number of private alleles among replicates
215 was calculated. DivMigrate was used to calculate migration rates between sampling sites and to assess
216 asymmetric migration patterns. A large number of loci (>1000) can partly compensate for the low
217 sample sizes (Willing et al., 2012), but results for populations with n < 5 should be interpreted with
218 caution (Sundqvist et al., 2016). To test the hypothesis that small in-steam barriers generally increase
219 fragmentation compared to reference sites and that weirs have a stronger effect than pipes or tunnels
220 (both culverts), linear mixed-effect models (LMMs) were run using the R-packages lme4 (Bates et al.,
221 2015) and lmerTest (Kuznetsova et al., 2017). First, mean differentiation (F_{ST}) for reference sites and
222 across barriers were calculated per barrier and species. Different LMMs were run for each species with
223 differentiation (F_{ST}) as dependent variable, presence of barriers between compared sites as fixed effect
224 and barrier site as random effect. In a second LMM analysis, barriers were subdivided into barrier types,
225 i.e. weirs, pipes, and tunnels (and no barrier) and compared per species.
226
227 **Population genetic simulations**
228 To simulate expected population differentiation caused by migration barriers over time, Nemo 2.3.51
229 (Guillaume & Rougemont, 2006) was used. As there is only little information available about the
230 parameters needed to be given in the simulation, various barrier strengths and biological species traits
231 were applied, including different mating systems (random mating for *G. fossarum* and hermaphrodite
232 for *A. fluviatilis* and *D. gonocephala*), population sizes, extinction rates, selfing rates for hermaphrodites
233 and dispersal and barrier models (see Figure 3 and Appendix S2 for details on parameter space). The

234 dispersal and barrier models were designed to reflect stream dispersal (linear, influenced by flow
235 direction) and our sampling scheme (four equidistant sampling sites). To prevent edge effects (no further
236 dispersal possible for edge populations in the simulation), we simulated six populations but only
237 analysed the four target populations. Simulations were run for 250 generations. The first 100 generations
238 were simulated with different dispersal models but without a barrier to separate dispersal model and
239 barrier effects. After 100 generations, a barrier was introduced for the next 150 generations using three
240 models (complete barrier, upstream barrier with unchanged, or reduced downstream dispersal, see
241 Figure 3 for details) and fstat files were saved every five generations.

242 Data analyses were conducted using different python and R-Scripts in a Snakemake workflow.
243 Edge populations were excluded and ten individuals per population were randomly chosen to reflect
244 average number of specimens sampled per population. Populations with fewer than ten individuals at
245 specific time points were excluded. Pairwise F_{ST}, H_O and AR were calculated for all populations every
246 five generations for each parameter combination, as described for ddRAD data. F_{ST} values were
247 calculated for all single populations and for pooled populations on both sides of the barrier. To evaluate
248 the performance of divMigrate in the detection of reductions of migration rates due to barriers, a subset
249 of output files were generated with the migration model "*asymmetric15*" and all barrier models (except
250 upstream barrier with reduced downstream 5%) for both mating systems and population sizes of 100
251 (500 for reduced downstream dispersal models) and 1000 with extinction rates of 0 or 0.6. Migration
252 rates (using G_{ST}) were calculated at different time points before (5, 50 and 95 generations) and after
253 barrier introduction (after 110, 150, 200 and 250 generations).

254

255 **Results**

256 All five macroinvertebrate species showed strong regional and local population structure as detected by
257 all methods.

258 **Population structure of *Gammarus fossarum***

259 We found *G. fossarum* in nine streams at 11 barrier sites (Table S1, Tables S3 and S4 for summary
260 statistics for all species). PCA (Figure S2) and sNMF analyses indicated strong population structure. In

261 the sNMF analysis, cross-entropy (CE) was lowest for k = 9 (Figure S3A). The nine clusters
262 corresponded to the nine streams, also visible in the Neighbour-Net analysis (Figure 4A and B). Further,
263 the network showed superordinate clustering into three phylogenetically distinct groups in concordance
264 with sampling site topography. Pairwise F_{ST} values were generally high (mean 0.47), especially between
265 network-groups and all values were significant, including values for the comparison between barrier
266 sites in one stream, ranging between 0.01 and 0.74 (Figure 4C). Likewise, estimated migration rates
267 were very low in most cases (mean 0.05), with the exception of within-stream comparisons (Figure 4D,
268 Table S5A). However, for the two within-stream comparisons, migration rates between VR6 and VR17
269 were much lower in both directions than those between QB17 and QB23 and were significantly higher
270 in the downstream than in the upstream direction. In 71% of the comparisons, significant asymmetric
271 gene flow was detected.
272
273 **Population structure of *Dugesia gonocephala***
274 *D. gonocephala* was found at 13 barrier sites in 11 streams. Of 344 specimens, 294 were diploid and 50
275 were potentially triploid. PCA (Figure S2) and sNMF cluster analysis both indicated strong population
276 sub-structuring. CE was lowest for K = 13 (Figure S3B). The population structure could be explained
277 best by the stream origin and ploidy level (Figure 4E). At sites with both diploids and potential triploids
278 (QB24, QB17 and QB23 and VR9), individuals were divided into two clusters or showed high
279 intermixture between clusters. The Neighbour-Net showed a star-like pattern containing 11 clusters,
280 each representing a single stream (Figure 4F), but sub-clusters within streams according to ploidy level
281 were visible. F_{ST} values were lower than those for *G. fossarum* (mean: 0.31, range 0.08 to 0.51) but
282 significant for all pairwise comparisons including within stream comparisons (Figure 4G). In general,
283 estimated migration rates were higher than those for *G. fossarum* (mean: 0.16) and migration rates for
284 most comparisons were significantly asymmetric (73%; Figure 4H, Table S5B). Similar to *G. fossarum*,
285 migration rates were highest between sites within a stream and migration was significantly higher in the
286 downstream direction.
287

288 **Population structure of *Ancylus fluviatilis* I, II and III**

289 Among 389 specimens, 167 were assigned to *A. fluviatilis* I, 159 to *A. fluviatilis* II and 63 to *A.*
290 *fluviatilis* III. In contrast to *G. fossarum* and *D. gonocephala*, H_O, F_{ST} and F_{IS} values varied strongly
291 between stacks settings (Table S4). For *A. fluviatilis* I, PCA (Figure S2) and sNMF indicated strong
292 genetic structure. CE values were low k = 7–10 (Figure S3C), with the lowest mean CE over repetitions
293 for k = 8. The clusters mainly corresponded to streams, but more specimens showed higher admixture
294 to additional clusters (Figure 5A). These results corresponded well with the network results (Figure 5B),
295 where a similar group structure consistent with streams was visible, with the exception of specimens
296 from QB12 and QB20, which were split into two groups according to the barrier site. In general, pairwise
297 F_{ST} values were lower than those in *G. fossarum* and *D. gonocephala* (mean: 0.18, range: 0 – 0.29,
298 Figure 5C), but all were significant with the exception of the within-stream comparison QB11 and
299 VR20. Migration rates were between the two previously described species (mean: 0.11), with 80% of
300 comparisons showing significant asymmetry (Figure 5D, Table S5C).

301 For *A. fluviatilis* II, PCA (Figure S2) and sNMF both indicated strong population structure
302 within the species. The most probable number of clusters in the sNMF analysis was k = 8 (Figure S3D).
303 The clusters were less clear than those for the other species, as many specimens showed high amounts
304 of shared ancestry to different clusters. The main factor explaining the groups was stream origin (Figure
305 5E), but in some cases, main cluster were shared among different streams or additional cluster were
306 found within one stream. A similar structure was observed in the Neighbour-Net analysis. However,
307 single specimens with high admixture in the sNMF plot, grouped together with specimens sampled in
308 other streams (Figure 5F). Overall, pairwise F_{ST} values indicated relatively low but significant
309 differentiation between all sampling sites, except for QB23–QB24 (mean: 0.08, range: 0.003 – 0.15,
310 Figure 5G). Estimated migration rates were higher than those for the other species (mean: 0.17) and less
311 asymmetry was detected (58% of comparisons, Table S5D).

312 *A. fluviatilis* III was mainly found in one stream with two barrier sites (VR6 and VR17). At
313 VR9, four individuals were identified as *A. fluviatilis* III/I hybrids (75%/25%) according to Weiss et al.
314 (2018) and these were included here. In the PCA (Figure S2) and sNMF analysis, sub-structure was

315 detected, with four clusters best explaining the population structure (Figure S3E). One cluster consisted
316 of VR9, the second of VR17 specimens and the other two of VR6 specimens, with shared proportions
317 for some individuals between clusters (Figure 5I). A similar pattern was observed in the Neighbour-Net
318 analysis (Figure 5J). Pairwise F_{ST} values were all significant but lower between VR6 and VR17 (Figure
319 5K), where also higher migration rates were detected (Figure 5L, Table S5E).

320 For all species, we detected a correlation between geographic and genetic distances, which was
321 strongest for *A. fluviatilis* II and weakest for *D. gonocephala* (Figure S4). Genetic diversity (i.e., H_O and
322 AR) differed among sites for all species, but with no consistent pattern over all species (Table S6).
323

324 **Effects of in-stream barriers on population structure**

325 Filtering loci separately for each barriers site, resulted in in a greater number of variable loci and mostly
326 increased genetic diversity per barrier site in comparison to the total dataset (Table S7). We could not
327 detect distinct barrier effects across species when comparing F_{ST} values between reference and barrier
328 separated sites. Mean differentiation was low and mostly insignificant regardless of the presence of
329 barriers or the barrier type (weir, pipe or tunnel). Consequently, none of the LMMs indicated a general
330 effect of barriers on differentiation (Appendix S3). When studying specific barriers in detail, we found
331 no evidence for a barrier effect on population structure for seven of the nine weirs and six of the eight
332 culverts in any species. This means that populations at barrier sites were either i) not differentiated at
333 all, ii) differentiation was similar among reference and barrier separated sites, iii) lower for some of the
334 comparisons of barrier separated sites compared to reference sites, or iv) that only individual sampling
335 sites were constantly differentiated to the other sites from the same barrier site. All these four
336 differentiation patterns were found for all species. Differentiation between individual sites was most
337 prominent for specimens sampled in affluents ("N"; Figure 6), despite the lack of obvious barriers. At
338 three weirs, reduced upstream migration rates were detected for *A. fluviatilis* I and II (QB11, QB12 and
339 QB17) (Figure S5). However, we found no indication for a barrier effect in any of the other analyses. A
340 similar reduction was observed for VR6a for *D. gonocephala* but here the sample size was too small at
341 upstream sites (Figure S5).

342 We only detected distinct barrier effects associated with weirs for *D. gonocephala* at QB24 and
343 QB11. In both cases, differentiation across barriers was larger than that between reference sites (Figure
344 6). The effect was stronger for QB24 than for QB11, consistent with the sNMF results, where two
345 clusters were the most probable for QB24. Specimens sampled upstream of the barrier only belonged to
346 the first cluster, while specimens assigned to both clusters, as well as intermixed ones, were found
347 downstream of the barrier. Specimens showing high membership to the second cluster were all classified
348 as potential triploids. At both barriers, migration rates in up- and downstream directions were lower
349 across barriers than among reference sites. For QB24, all rates across the barrier were significantly
350 asymmetric, with stronger downstream dispersal. Also, genetic diversity was higher downstream of
351 barriers at both sites, indicated also by higher amounts of private alleles (Tables S8 and S9).

352 A significant barrier effect was detected for *A. fluviatilis* II at VR20 and for *A. fluviatilis* III at
353 VR6b. While VR6b was a pipe, VR20 was a combination of a pipe and tunnel with an additional steep
354 drop within and two streams were joined within the tunnel. At VR20 S4, we only found one specimen
355 belonging to *A. fluviatilis* II, which was excluded from analyses of F_{ST} and migration rate. The other 11
356 specimens at this site were identified as *A. fluviatilis* I, which was not detected upstream of the culvert.
357 All individual site comparisons across the culvert in *A. fluviatilis* II indicated significant differentiation,
358 including comparisons of both N1 and N2 with S2, which were separated by a pipe and part of the
359 tunnel, while differentiation among reference sites was not significant. This pattern was also detected in
360 the divMigrate analysis. Upstream migration across the culvert to both ends was highly reduced, while
361 downstream migration rates were similar or even higher than those among reference sites (Figure S5).
362 In the sNMF analysis, indications for a barrier effect were found with the lowest CE for three clusters.
363 Specimens from both N1 and N2 were split into two clusters (k1: n = 10, k2: n = 6), S1 specimens were
364 assigned to k2 or the third cluster (k2 = 6, k3=5) and specimens at S3 were assigned to all three clusters,
365 indicating that downstream dispersal was possible. AR was lowest at S2; otherwise, genetic diversity
366 was similar (Tables S8 and S9).

367 At barrier site VR6, the longer pipe of this barrier site (VR6b) caused population differentiation
368 in *A. fluviatilis* III, as all comparisons across the pipe were significant and F_{ST} values were higher than

369 those between reference sites (Figure 6). These differentiation patterns were supported by the sNMF
370 analysis, where three clusters were detected. One cluster was only found in S3&4, while the other two
371 were mainly found in S1&2 and less frequently in S3&4, where assignment to multiple clusters was
372 observed. While migration rates between sites were generally reduced, upstream migration across the
373 second pipe (VR6b) was even stronger reduced to nearly zero. By contrast, migration rates across the
374 first pipe (VR6a) were even higher than those among reference sites (Figure S5). H_O was similar at all
375 sampling sites, while AR and the number of private alleles were higher at S3&4 than at other sites
376 (Tables S8 and S9).
377 Unlike in *D. gonocephala* and *A. fluviatilis*, we did not detect evidence for a barrier effect on
378 population structure in *G. fossarum* at any of the barriers.
379
380 **Population genetic simulation**
381 The simulation of a complete barrier revealed the quick emergence of strong population differentiation,
382 as determined by increases in F_{ST} values and decreases in migration rates over time (Figure S6, Table
383 S10). In general, barrier effects were similar, independent of the distance between populations. In
384 smaller populations, fluctuations over time were larger, leading to low but significant population
385 differentiation between reference populations not separated by a barrier. However, after only a few
386 generations, F_{ST} values for populations separated by a barrier were consistently higher than those
387 between reference populations. For larger populations (i.e. patch capacity 2000), it took up to 50
388 generations to detect differentiation and F_{ST} values remained low but followed the same general pattern
389 (Figure S6). Here, only F_{ST} values between populations separated by a barrier were significant. The
390 higher the extinction rate, the higher the F_{ST} values between barrier-separated populations and also in
391 larger populations, differentiation was detected earlier. For smaller populations with higher extinction
392 rates, significant differentiation was detected without a barrier but remained lower than for barrier-
393 separated populations. These patterns were visible for all tested migration matrices, with asymmetric
394 matrices leading, on average, to slightly higher F_{ST} values. However, while a reduction of migration
395 rates over time was detected with the divMigrate approach, the simulated asymmetry was not reflected

396 in consistent differences between up- and downstream migration rates. Applying different selfing rates
397 to simulate hermaphrodites, F_{ST} values increased with increasing selfing rates, but patterns were
398 generally the same as those for populations with obligate sexual reproduction. Even though complete
399 isolation between populations on both sides of the barrier was not directly detected, a reduction in
400 migration rates after barrier introduction was quickly (<10 generations) detected for all migration
401 scenarios and decreased further with time. The reduction was more easily detected for larger sample
402 sizes, higher extinction rates and smaller populations.

403 In general, barriers impeding upstream but not downstream dispersal did not affect the population
404 structure and did not result in reduced inferred migration rates (Figure S6, Table S10). At some time
405 points for small populations (particularly in combination with higher extinction and/or selfing rates),
406 F_{ST} values were slightly higher and significant for barrier-separated populations than for reference
407 populations, but this pattern fluctuated over generations and did not increase with time (similar for
408 migration rate estimates). Reducing downstream dispersal rates led to an intermediate pattern between
409 those described previously. Not all parameter combinations were simulated, but downstream dispersal
410 rates had to be reduced substantially to 1 %, or depending on the other parameters 2 %, to influence
411 population differentiation. The effects were stronger (and sometimes only detectable) for smaller
412 population sizes, higher extinction or selfing rates, and were strongest for a combination of these
413 parameters (i.e., if population sizes were small and extinction rates were high). If the selfing rate was
414 increased from 0.3 to 0.6, the effect on the population structure was generally high but barrier effects
415 were less detectable because fluctuations between populations and over generations were higher. We
416 obtained similar results for migration rates, but correct asymmetry was not reliably detected. H_O and AR
417 were, in general, not affected by the barrier model (Figure S7). Both AR and H_O decreased stronger over
418 time for smaller population sizes and higher extinction proportions for all dispersal models (with no or
419 slow decreases for larger population sizes, i.e., 1000 and 2000). However, the decrease was constant
420 over time, independent of the introduction of a barrier into the system and similar in all populations.
421
422

423 **Discussion**

424 **General effects of small in-stream barriers on macroinvertebrate taxa**

425 For all five stream species, our population genomic data revealed strong population differentiation at
426 local scales. However, in contrast to our hypothesis, the tested individual small barriers did only increase
427 genetic differentiation compared to control sites in few cases. No correlation between barrier strength
428 (weir, pipe or tunnel) and differentiation was found. Therefore, we found little evidence that the tested
429 in-stream barriers are the cause of the observed local scale population isolation. Only for few individual
430 in-stream barriers we detected a significant barrier effect: two weirs for *D. gonocephala* and one culvert
431 each for *A. fluviatilis* II and III. This raises the questions i) whether barrier effects are detectable with
432 our chosen methods for barriers with an age of 33-109 years and ii) whether population genetic
433 signatures indicate fragmentation if only upstream dispersal is impeded. To address both questions, we
434 simulated population genetic null models under specific life history traits and explored life-cycle and
435 barrier parameter space as realistic as possible. However, greater knowledge on the biology of the
436 species would be needed to obtain more precise null models for comparison as otherwise endless
437 possibilities for parameter combinations exist.

438 The simulation results consistently showed that barriers preventing up- as well as downstream
439 dispersal lead to strong population structure relatively quickly within the age range of the barriers tested.
440 Other studies simulating the effect of barriers on population structure found that barrier effects will only
441 be detectable with F_{ST} after longer times (Landguth et al., 2010), or for small populations (Ne < 100;
442 Coleman et al., 2018), but simulation parameters were quite different in these simulations, for example
443 different sampling schemes or marker were used, making it difficult to compare simulations. Here, we
444 found that the time until effects were detectable depended on the population size and extinction rates,
445 with smaller populations showing barrier-related structure already after 5 to 20 generations and the
446 largest populations (patch capacity 2000) after approximately 50 generations.

447 Our simulations indicated that F_{ST} was the most reliable indicator of fragmentation. H_O or AR
448 could not be used as both decreased constantly over time in small reference and barrier-separated
449 populations, or remained relatively constant in larger populations. With migration rate estimates by

450 divMigrate, complete barriers were detectable as well by a general reduction in migration rates between
451 barrier-separated sites. However, in general, migration rates were overestimated, suggesting that
452 inferred low migration rates in real data probably indicate real barrier effects.

453 To conclude, simulations showed that even minor migration (2 % or 5 % depending on population
454 size, extinction and selfing rate) in one direction can be sufficient to counteract barrier-related
455 population differentiation. If populations are large (patch capacity >2000) and no extinction rates are
456 simulated, even 1 % downstream migration can be enough to counteract differentiation in random
457 mating populations. Accordingly, reduced migration due to barriers is expected to be detectable if
458 migration rates are low, but detection will be difficult or impossible for very young (<10 years) barriers,
459 for barriers only reducing upstream dispersal and for large effective population sizes.

460

461 **Species-specific barrier effects**

462 We hypothesised that effects would depend on barrier strength and would be strongest for *A. fluviatilis*,
463 intermediate for *D. gonocephala* and lowest for *G. fossarum*. Our results only partly support the
464 hypotheses as only single species at a few strong barriers we detected a stronger population structure
465 and reduced migration rates compared to those for reference populations. For most weirs and culverts,
466 no barrier effect was found. This indicates that all species can in principle overcome the tested small in-
467 stream barriers frequently enough (or at least in one direction) to facilitate gene flow. However, it cannot
468 be ruled out that barrier effects were not detectable even though they exist because of too large effective
469 population sizes or because barriers only reduced upstream dispersal as discussed above. Additionally
470 to active dispersal, hololimnic specimens may overcome barriers by passive dispersal via animal vectors
471 such as waterfowl. This process can be frequent at local scale (Coughlan et al., 2017) and experimental
472 evidence suggests that it could be an important dispersal mechanism for amphipods, snails and other
473 invertebrates (Rachalewski et al., 2013; van Leeuwen & van der Velde, 2012; Waterkeyn et al., 2010).
474 While no information is available on the probability or frequency of zoochorous dispersal for the taxa
475 studied here and in general more information is needed, e.g. on the quantitative contribution of zoochory

476 to dispersal (Coughlan et al., 2017), it should be considered as a possible mechanism counteracting
477 barrier effects.

478 With respect to the individual species, the barrier effects were weakest for *G. fossarum* clade 11,
479 as expected. This indicates that dispersal ability is high enough to overcome the small in-stream barriers
480 analysed here (see also Weiss & Leese, 2016), congruent with findings for *G. fossarum* clade 12 (Alp
481 et al., 2012). It is also possible that the tested barriers were not old enough to create detectable isolation
482 patterns (Monaghan et al., 2001), that the reduction in migration was not strong enough to impact
483 population structure determined by the genetic markers (Whiterod et al., 2017), or that effective
484 population sizes were too large as *G, fossarum* is expected to have the largest N_e of all taxa tested here.
485 However, based on the barrier ages (>30 years, ≥ 30 generations), distinct local population structure,
486 simulation results and the comparably high mobility of the species with the capability for active and
487 passive dispersal, we consider it more likely that the tested small in-stream barriers do not present severe
488 dispersal barriers for this species.

489 Our prediction that barrier effects would be moderate for *D. gonocephala* and strongest for *A.*
490 *fluviatilis* was not supported by the data. Only two weirs and two culverts had species-specific barrier
491 effects. The weirs influencing *D. gonocephala* were classified as the second (QB24) and third (QB11)
492 most severe according to height, steepness and smoothness. They were both >80 years old. For other
493 barriers of the same age, no population structure was detected as well as for QB27, which was even
494 higher than QB24 with a smoother ramp slope. The difference between barriers of the same age might
495 be explained by differences in population size or by important barrier characteristics that had not been
496 measured. The detected barrier effect was weaker for QB11 than for QB24 which was not as high and
497 had a moderate slope with drops at the start and the end. The two drops might have strengthened the
498 effect in comparison to other barriers with similar slope. In general, our results indicate that *D.*
499 *gonocephala* maintains gene flow across most small weirs, but we also found evidence that weirs of the
500 size and shape tested can impede migration. This holds true especially for those classified as more or
501 most severe. Thus, larger barriers, especially larger drops, will probably impact on dispersal for this
502 flatworm species. Contrary to our expectations, *A. fluviatilis* migration was not stronger affected by

503 weirs. At three weirs, upstream migration rates across barriers were lower than those between reference
504 sites, but no effect was detectable with any other method. To finally conclude that small weirs do not
505 influence migration in *A. fluviatilis*, it would be important to increase the sample size and to evaluate
506 the influence of polyploidy on analyses. In addition, especially larger drops should be investigated as
507 they will probably pose a greater barrier to dispersal than steep ramps, if they cannot be crossed by
508 crawling or passive zoochorous dispersal.

509 For culverts, patterns were more consistent with our expectations. We only detected effects for *A.*
510 *fluviatilis* II and III at two culverts predicted to have the strongest impact (VR20 and VR6b). At VR20,
511 only *A. fluviatilis* II was found upstream of the culvert, while *A. fluviatilis* I was additionally found at
512 downstream sites. While downstream dispersal seemed to be possible through this culvert, upstream
513 dispersal was highly limited. A similar pattern was found for *A. fluviatilis* III at VR6b, even though here,
514 inferred migration in both directions was reduced also between reference sites, but stronger across the
515 pipe. For the other species, we could not attribute observed population structure between sampling sites
516 to culverts, or the sample size upstream of the barrier was too small *(D. gonocephala*). These results
517 indicate that all studied species can probably disperse effectively through tunnels of up to 120 m length,
518 but pipes < 25 m can act as strong dispersal barriers probably due to their smooth internal structures and
519 high flow velocity (David et al., 2014), or drops at the end, preventing upstream movement (Vaughan,
520 2002). Also for culverts it is possible that barrier effects went undetected due to, e.g. large N_e. But in
521 general our results were in accordance with the expectations that tunnels present less severe barriers
522 than pipes, and it seems possible that the studied taxa can overcome them actively. However, with
523 respect to pipes our results suggest that these can pose severe dispersal barriers even when relatively
524 short. Therefore, it would be important to focus on this kind of culverts in further studies.

525 In general, single small barriers did not have a large impact on population structure, yet it remains
526 to be determined whether cumulative effects exist and population structure increases when many small
527 barriers occur within short distances. Future work should focus on testing and adapting novel and
528 promising indices to identify population fragmentation in streams developed based on microsatellites

529 (Prunier et al., 2020) to SNP-based data including also possible asymmetric gene flow and focus on
530 barriers at the upper level of the severity gradient tested here, for weirs as well as for culverts.
531
532 **Regional subdivision**
533 While effects of individual in-stream barriers was minor, genetic differentiation between streams was
534 high for all species despite the small spatial scale of the study. Based on life history traits, we
535 hypothesised that population structure to be weakest for *G. fossarum*, intermediate for *D. gonocephala*,
536 and greatest for *A. fluviatilis*. However, contrary to our expectation, *G. fossarum* showed the highest
537 population differentiation and the lowest migration rates between populations, *A. fluviatilis* species
538 showed the lowest and *D. gonocephala* showed an intermediate level of differentiation.
539 For *G. fossarum*, populations were already highly differentiated at the shortest distances of 11–
540 14 km, with F_{ST} values of approximately 0.35. Analysing populations sampled at sites approximately
541 2 km apart within a single stream showed that gene flow in *G. fossarum* is possible over this distance
542 but can already be reduced. In contrast to the other species, we also detected a strong phylogeographic
543 structure. Populations clustered into three groups in the network analysis, congruent with the geographic
544 distribution and mitochondrial COI groups defined by Weiss & Leese (2016), indicating that the area
545 was probably recolonised by at least three distinct historical source populations after Pleistocene
546 glaciation. This strong influence of historical processes on population differentiation could explain the
547 higher population differentiation in comparison the other species. Still, the strong differentiation after
548 10 km is inconsistent with the relatively high mobility, locally often large populations and regionally
549 broad distribution. It is possible that the dispersal ability is lower than expected or, more likely, that
550 unidentified obstacles to dispersal exist. These obstacles could be human-induced alterations and
551 fragmentations, such as larger dams, weirs and culverts (especially larger pipes), or unfavourable
552 conditions in connecting areas, such as those caused by anthropogenic land use, organic pollution,
553 acidification or large connecting rivers, or a cumulative effect of these factors (e.g., Alp et al., 2012;
554 Cook et al., 2007; Monaghan et al., 2001; Watanabe et al., 2010). Furthermore, gene flow does not
555 correspond directly with individual movement of specimens (e.g., Bohonak & Jenkins, 2003) when

556 dispersing individuals are not able to successfully establish in an existing population. This phenomenon
557 is described by the monopolisation hypothesis (e.g., De Meester et al., 2002), which predicts that
558 adaptation along with the numerical advantage of first migrants leads to a priority effect of the founder
559 population over new migrants and therefore reduces establishment success. Such intraspecific priority
560 effects as well as isolation by adaptation patterns have been detected in various taxa (e.g., Boileau et al.,
561 1992; Fraser et al., 2014; Nosil et al., 2008; Urban & De Meester, 2009) and could have led to increased
562 differentiation.
563 In *D. gonocephala*, we detected fairly high population differentiation, but in contrast to *G. fossarum*, no
564 strong geographic structure was found, supported by the low IBD pattern. Populations separated by only
565 approximately 2 km in the same stream already showed significant population differentiation.
566 Differentiation was also detected for 11 to 18 km but was not correlated with waterway distance within
567 this range, suggesting that additional factors other than waterway distance influence effective migration
568 in this species. Local adaptation could shape the population structure, as reported for a population in the
569 same study area in response to high copper concentrations (Weigand et al., 2018), in which also high
570 population differentiation was found. Apart from this, little is known about effective dispersal in *D.*
571 *gonocephala*, but planarians are generally been regarded as weak dispersers (e.g., Rader et al., 2017).
572 The overall lower F_{ST} values in comparison to *G. fossarum* could indicate that *D. gonocephala* is a better
573 disperser. However, it is more likely that differences in F_{ST} reflect the greater phylogenetic signal in *G.*
574 *fossarum*, represented by three deep phylogenetic lineages as described above. In *D. gonocephala*,
575 particularly strong population structure in some cases was associated with the presence of potentially
576 triploid individuals. Potential triploids originating from different streams did not cluster together but
577 were closely related to specimens in the same stream, indicating that polyploidy evolved independently
578 several times. However, results concerning possible polyploid individuals have to be interpreted with
579 caution, i) because it is difficult to assess how analyses are affected for example by allelic dosage
580 uncertainty (Dufresne et al., 2014) and ii) because we estimated ploidy levels only indirectly and cannot
581 say if individuals are really triploids or aneuploids with high chromosome numbers as it has been implied
582 for a French *D. gonocephala* population (de Vries, 1986). Therefore, chromosome numbers in respective

583 populations should be analysed by karyograms or flow cytometry. At sites with potential triploids, they
584 often made up the majority, consistent with previous results showing that asexual polyploids are more
585 abundant than diploids (Álvarez-Presas & Riutort, 2014). Asexual populations of *D. gonocephala* have
586 not been reported (Stocchino & Manconi, 2013), but we cannot exclude the possibility and polyploid
587 populations should be analysed in more detail in future studies.

588 The low genetic structure in *A. fluviatilis* species could be explained by the presence of three
589 relatively young cryptic species in the area (see Weiss et al., 2018 for details), as time since speciation
590 might have been not long enough to generate similar variation compared to e.g. the extremely diverse
591 *G. fossarum*. Further, analyses were complicated by the polyploid genomes. The chosen analysis
592 approaches should be able to generate reliable results concerning population genetic structure (Dufresne
593 et al., 2014), but also loci detection could have been influenced by the ploidy. Even though we chose
594 the most rigorous Stacks settings to disentangle homoeologous loci of different genomes (as defined in
595 Dufresne et al., 2014), observed heterozygosity, particularly for *A. fluviatilis* II, was still much higher
596 than for *D. gonocephala* and *G. fossarum*. Even though this filtering was necessary, it led to fewer loci
597 being analysed for the different *A. fluviatilis* taxa. Despite the weaker population structure, significant
598 differentiation between populations originating from different streams and generally low migration rates
599 among populations were detected for all *A. fluviatilis* species and a high number of genetic clusters was
600 supported by the sNMF analyses. Additionally, most within-stream comparisons showed low but
601 significant differentiation, even within a few hundred meters. In general, our findings suggest that
602 effective dispersal is low between streams and differentiation within streams can occur over short
603 distances (see also Macher et al., 2016). In some cases, we detected genetic structure even within barrier
604 sites, while in other cases, levels of shared ancestry between clusters were higher than those for *G.*
605 *fossarum* or *D. gonocephala*. This may reflect higher occasional gene flow between streams, a younger
606 recolonisation history, or could have been influenced by polyploidy and the still high heterozygosity in
607 the data set. For a better understanding of the determinants of population structure, it is important to
608 better characterise homoeologous loci and to determine ploidy levels of individuals. However, the
609 consistent overall patterns across species suggest that our results are reliable and population isolation

610 despite differences in dispersal capacity indicates that strong dispersal limitations exist in the area, which
611 are not identified, yet.
612
613 **Conclusions**
614 We detected strong genetic isolation among populations of five hololimnic species between streams.
615 While for some individual barriers and species an effect was found, our genomic ddRAD data suggest
616 that single small weirs and culverts are probably not the cause for the detected strong fragmentation at
617 few kilometres distances. It remains to be tested, if cumulative effects of small barriers could have
618 caused the disruption of gene flow between populations, or if other factors, such as larger in-steam
619 barriers, land use, chemical pollution, urbanisation, or a combination of these, led to the observed
620 population structure. In general, our combination of genomic markers, population genetic simulations
621 and a controlled sampling design with distinct reference populations is a suitable tool to infer major
622 drivers of regional and local population structure.
623
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632
633 **Data Accessibility**
634 ddRAD data for *G. fossarum* and *D. gonocephala* will be available at NCBI BioProject upon publication.
635 ddRAD data for *A. fluviatilis* I, II and III will be available at NCBI BioProject with accession number
636 PRJNA389679.

Conflict of interest

The authors declare that they have no competing interests.

Author Contributions section

MW, HW and FL designed the study. MW and HW collected the samples and wrote the scripts for ddRAD data analyses. MW performed the laboratory work and data analysis. MW and FL interpreted the data. MW wrote the manuscript and all authors contributed to the final version of the manuscript.

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Tables

Table 1. Barrier characteristics of culverts (VR) and weirs (QB), sorted by expected severity from high to low. First three VRs were categorized as pipes, all other were tunnels.

Name VR/QB	Approx. construction year	Approx. length VR/ height QB [m]	Approx. width × height VR [m]	VR bottom substrate/ QB quality structure	Description of VR/ steepness of QB ramps (low/med/high)
VR20	1906 (enlarged 1980)	94/114	tunnel: 2.5 × 1.2 pipe: ø 0.6	concrete	tunnel (94 m) between N2 and S3 with a steep drop; S2 joins tunnel via ~30 m-long pipe over small ramp upstream of drop; total length S1–S3: 114 m
VR6b	1960	22	ø 0.5	concrete	3 parallel concrete pipes, small drop at end
VR6a	1960	8	ø 0.5	concrete	2 parallel concrete pipes, small drop at end
VR17	1980	120	3 × 1	artificial sediment	broad tunnel, small drops between S1 & S2
VR23	1910 (enlarged 1970)	70	0.6 × 1	natural sediment	tunnel
VR12	–	38	1 × 1.2	concrete	tunnel
VR11	1970 (enlarged 1980)	34 (72)	3 × 2	concrete	big tunnel, up- & downstream 7 small drops
VR9	1980	56	3 × 4	natural sediment	big tunnel
QB27a	1930	1.65		smooth	high
QB24	1930	1.5		rough	high
QB11	1930	1.35		rough	medium, drops at start and end
QB12	–	1.1		rough	1st half medium, 2nd high
QB27b	1930	1.05		smooth	low, drops at start and end
QB22	1905	1.3		rough with bigger stones	medium
QB23	1930	1.2		rough with bigger stones	medium
QB17	1960	0.6		rough	medium
QB20	–	1		rough	low, small drop at end

872 **Figure captions**

873 Figure 1. Summary of traits differing among studied species. Numbers from 1 to 3 indicate the
874 expected relative gradient. The arrow indicates the expected strength of the barrier effects and
875 population structure.

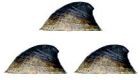
876 Figure 2. Central map showing the locations of barrier sites (QB = weirs, VR = culverts). Pie charts
877 indicate sampled species. Water course of sampled streams and main rivers are highlighted. Boxes
878 show the sampling scheme and locations of individual sampling sites, with a distance of approx. 200
879 m. Water flow direction is given by small arrows.

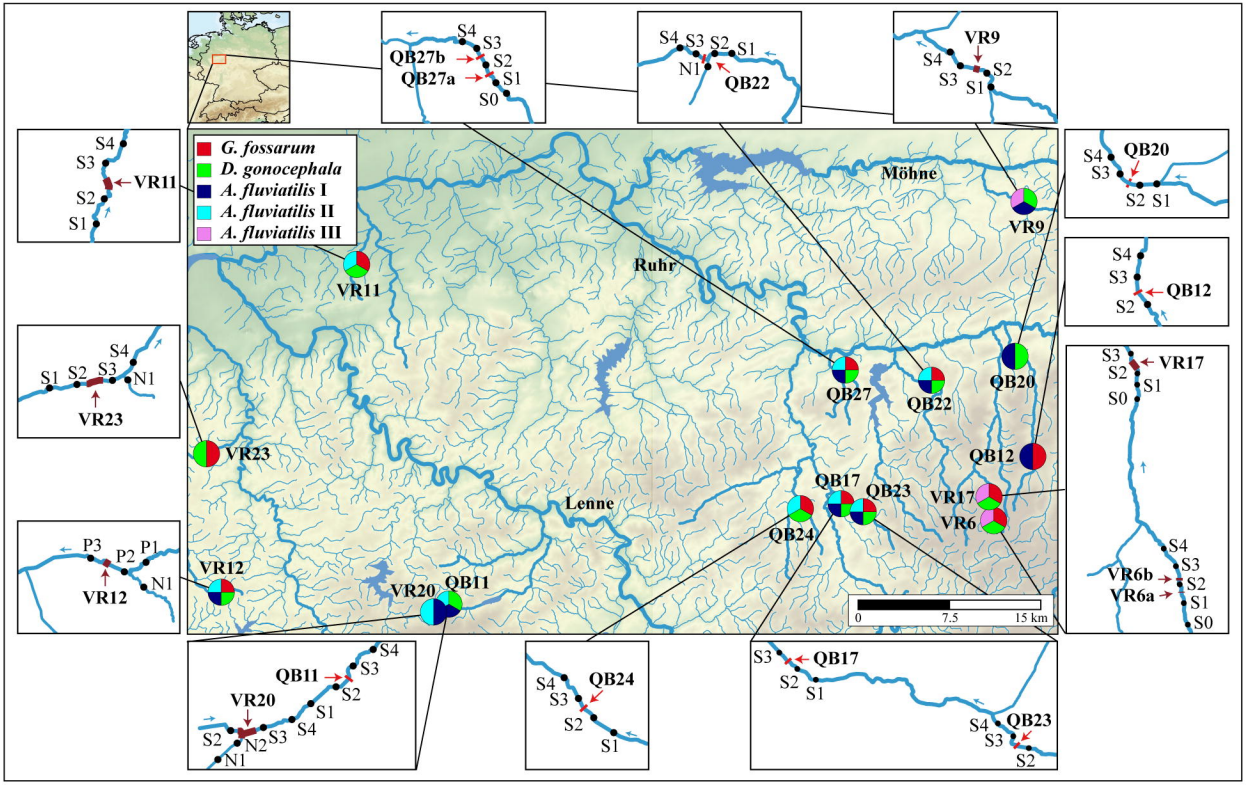
880 Figure 3: Workflow of the study. Left panel: Number of specimens analysed per species, sampling
881 scheme for a barrier site, summary of lab work, bioinformatic and statistical analyses. For general
882 population structure analysis per species (step 6), all specimens sampled at individual sampling sites
883 before and after the barrier were pooled (barrier site). Right panel: Details on population genetic
884 simulation including all important parameters and parameter space chosen for varied parameters and
885 data analysis steps.

886 Figure 4. Population genetic structure of *G. fossarum* (A-D) and *D. gonocephala* (E-H). A, E)
887 Ancestry estimates for A) K = 9 and E) K = 13, where vertical bars represent individual ancestry
888 coefficients. B, F) Neighbour-Net, branch colours indicate barrier sites. C, G) F_{ST} heat maps for
889 pairwise comparisons between barrier sites. Above the diagonal pairwise F_{ST} values are given and
890 below the lower confidence interval (values >0 indicate significant differentiation), all coloured
891 according to the level of differentiation. D, H) DivMigrate plot, strength and visibility of arrows are
892 correlated with migration rates.

893 Figure 5. Population genetic structure of *A. fluviatilis* I (A-D), II (E-H) and III (I-L). A, E, I) Ancestry
894 estimates for A, E) K = 8, I) K= 4, where vertical bars represent individual ancestry coefficients. B, F,
895 J) Neighbour-Net, branch colours indicate barrier sites. C, G, K) F_{ST} heat maps for pairwise
896 comparisons between barrier sites. Above the diagonal pairwise F_{ST} values are given and below the
897 lower confidence interval (values >0 indicate significant differentiation), all coloured according to the
898 level of differentiation. Non-significant values and corresponding CI are indicated in italics. D, H, L)
899 DivMigrate plot, strength and visibility of arrows are correlated with migration rates.

900 Figure 6. Pairwise F_{ST} values between all sampling sites for each barrier and species, coloured
901 according to presence of barriers. F_{ST} values from site stream populations (N) are shown in different
902 colours. Barriers are ordered according to severity, starting with the strongest weir and strongest
903 culvert. Combi: differentiation across both barriers at one barrier site. Barriers where an effect was
904 detected are indicated with an asterisk.

species	feeding type	reproduction / ploidy	relative population size	relative dispersal capability	predicted strength of barrier effect & population structure
G. fossarum	mainly shredder, (minor also grazer/collector, predator)	sexual / diploid	can have large local populations **1**	dispersal by swimming/ walking, often found in the drift **1**	least affected by barriers & lowest population structure **1**
D. gonocephala	predator	hermaphrodite, sexual / diploid or aneuploid	as predator smallest population size expected **3**	dispersal by crawling/ swimming possible, rarely found in the drift **2**	mid-level barrier effect & medium population structure **2**
A. fluviatilis I, II, III 	grazer	hermaphrodite, sexual or asexual / polypoid (different levels possible)	can have large local populations, but smaller than *G. fossarum* **2**	dispersal by crawling/ rarely found in the drift **3**	strongest barrier effect & highest population structure **3**

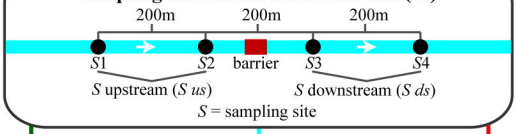

G. fossarum
D. gonocephala
A. fluviatilis I
A. fluviatilis II
A. fluviatilis III
Möhne
Ruhr
Lenne
VR11
VR23
VR12
VR20
QB11
QB27
QB22
QB20
QB12
QB17
QB23
QB24
VR17
VR6
VR9
QB27b
QB27a
QB22
QB20
QB12
VR17
VR6b
VR6a
QB11
QB24
QB17
QB23
0
7.5
15 km

D. gonocephala (Dg) n = 344

A. fluviatilis (Af) n = 392

G. fossarum (Gf) n = 345

sampling scheme for a barrrier site (*bs*)



labwork

DNA extraction (*Dg*, *Af*, *Gf**1)

↓

ddRAD library preparation (*Dg*, *Af**2, *Gf*)

↓

High-throughput sequencing (*Dg*, *Af*, *Gf*)

*1DNA extracts from Weiss & Leese 2016; *2ddRAD data of 269 *ind* from Weiss et al. 2018

ddRAD data analysis

1) **preprocessing** of data for each sequencing library
2) **loci identification** with Stacks: 8 settings per species
3) **ploidy level analysis** based on allele coverage distribution
4) **evaluation of stacks settings and filtering settings:**
   - calculation of global H_O, H_S, H_T, F_{ST} & F_{IS}
   - cluster analysis: PCA and sNMF (k=1-15)
   - comparison of results & decision on final filtering settings
5) **assignment** of individuals to ***Af* I**, II, III using sNMF results

6) **population structure analysis** per species (**population** = ***bs***)
   - calculation of global H_O, H_S, H_T, F_{ST} & F_{IS}
   - pairwise F_{ST}, H_O, allelic richness (AR)
   - PCA and sNMF (*Dg*, *Gf*: k=1-17; *Af* I, II, III: k=1-10)
   - Neighbor-Nets, migration rates (divMigrate)

7) **barrier analysis per *bs*** (**population** = ***S*** or ***S us*|*S ds***)
   - calculation of global H_O, H_S, H_T, F_{ST} & F_{IS}
   - pairwise F_{ST} between all *S* and between *S us*/ *S ds*
   - H_O, AR, # private alleles per *S* and *S us*/ *S ds*
   - sNMF (k = 1-6), migration rates (divMigrate)

population genetic simluation

Aim: generate null models for population differentiation caused by migration barriers over time by varying biological species traits unknown/variable between sites for the analyzed taxa.

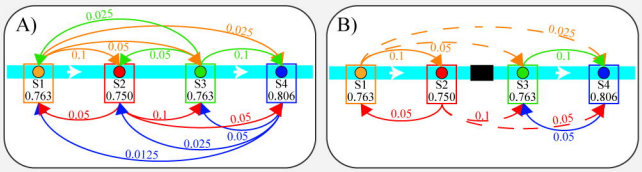
constant parameters

- 6 populations, only middle 4 analyzed to evade edge effects
- mean fecundity: 25; loci: 2000, 2 alleles/locus
- total duration: 250 generations (*gen*), file saved every 5 *gen*

varied parameters

- **mating system**: random mating / hermaphrodite (*herm*)
- **population size**: 100, 500, 1000, 2000; *herm*: + 50 and 200
- **extinction rate**: 0.0, 0.6, 0.8; *herm*: **selfing rate**: 0.0, 0.3, 0.6
- **dispersal models** (*dm*):
  - *asymmetric10*: 5% *us*/10% *ds* dispersal to next population, proportions to further populations always haved (see figure)
  - *asymmetric15*: 7.5% *us*/15% *ds*, then dispersal rates halved
  - *symmetric10*: 10% *us/ds*, then proportions halved

\+ **barrier model** (*bm*): barrier after 100 *gen* under *dm*:
  - *complete barrier*: no *us/ds* dispersal across barrier (all *dm*)
  - *upstream barrier*: no *us* dispersal barrier (all *dm*); *ds* either as without barrier (all *dm*) or reduced to 1%/2%/5% to next population, then rates halved (only *dm asymmetric15)*



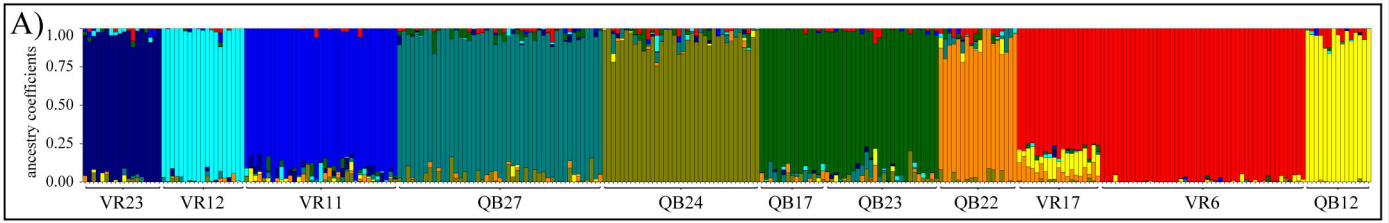
dm asymmetric10 for targed populations *S*1-4: numbers at arrows are dispersal rates; numbers in squares are (rounded) resident proproportions A) *dm* without barrier (100 generations) B) *dm* with barrier (+ 150 generations); solid lines: dispersal under all *bm*; dashed lines: additional dispersal for upstream *bm*

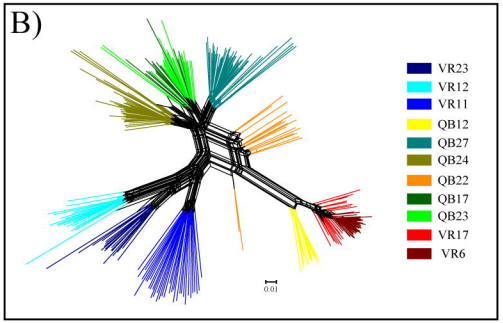
simulation data analysis

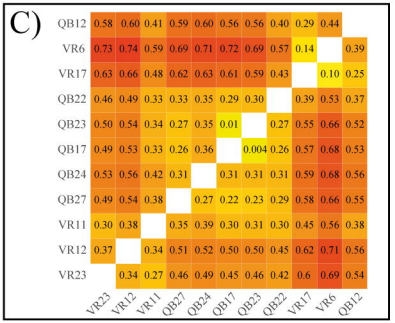
- pairwise F_{ST} values between all *S* and *S us* / *S ds*
- H_O and AR for all *S*
- plots for F_{ST}/ H_O / AR over time for all parameter settings
- divMigrate analyses for selected time points and settings

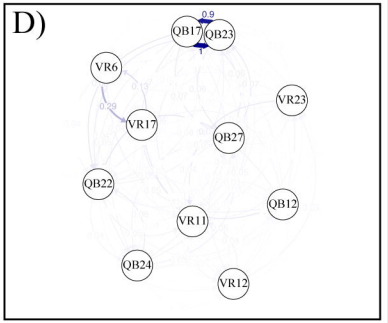
Using null models generated by simulations to interpret barrier effects found with ddRAD data analysis

G. fossarum

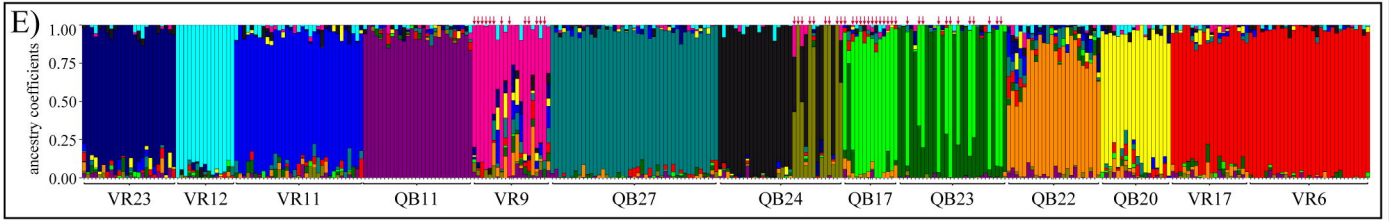


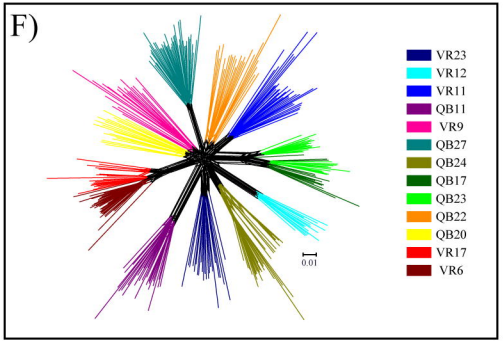


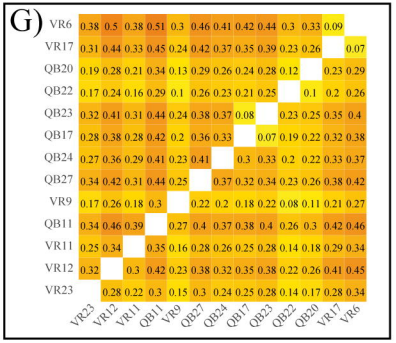


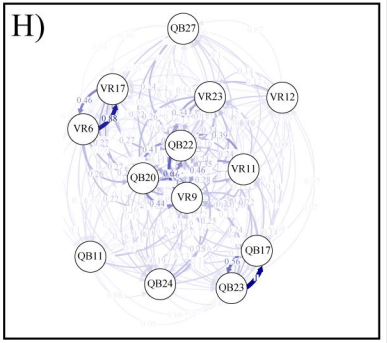


D. gonocephala

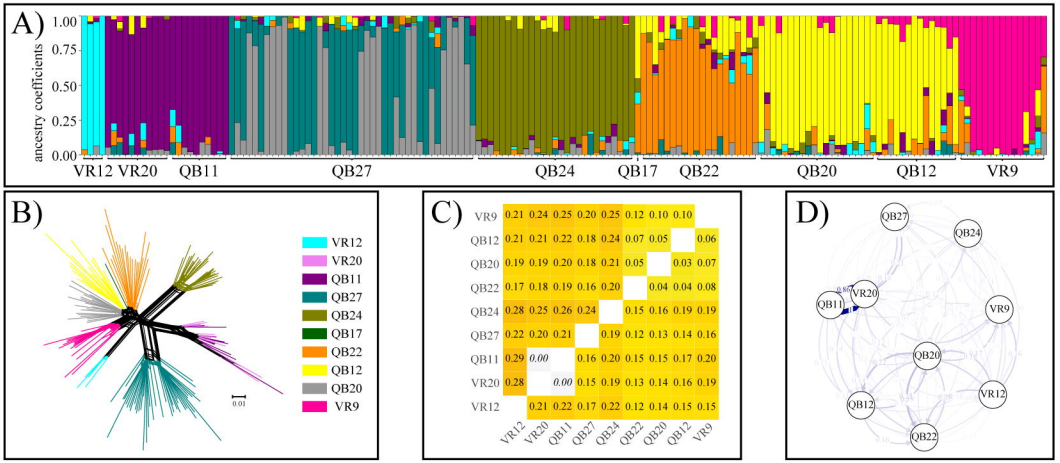




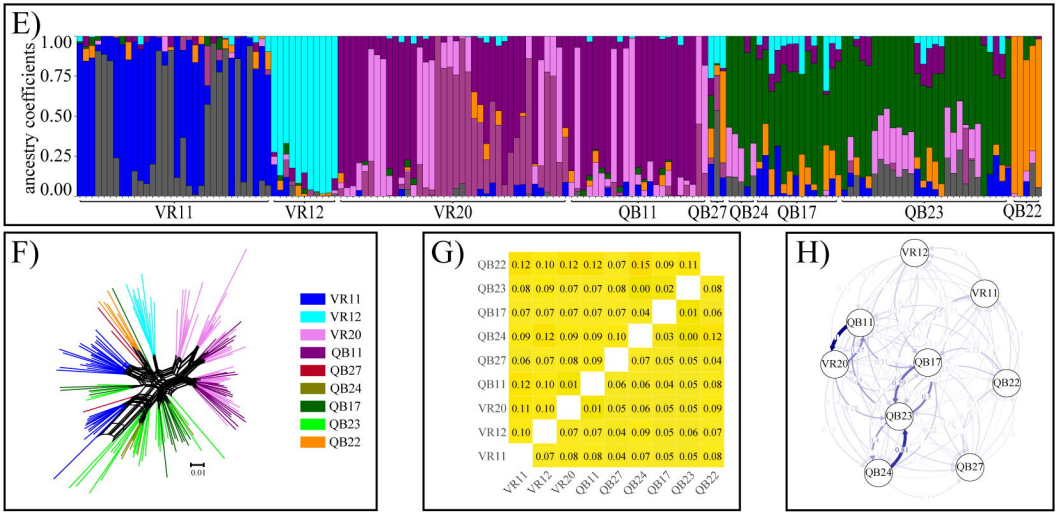




A. fluviatilis I



A. fluviatilis II



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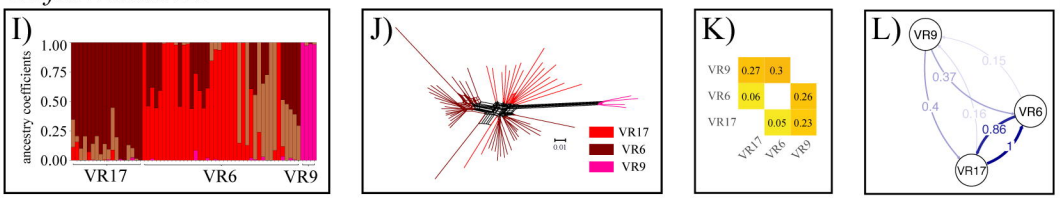

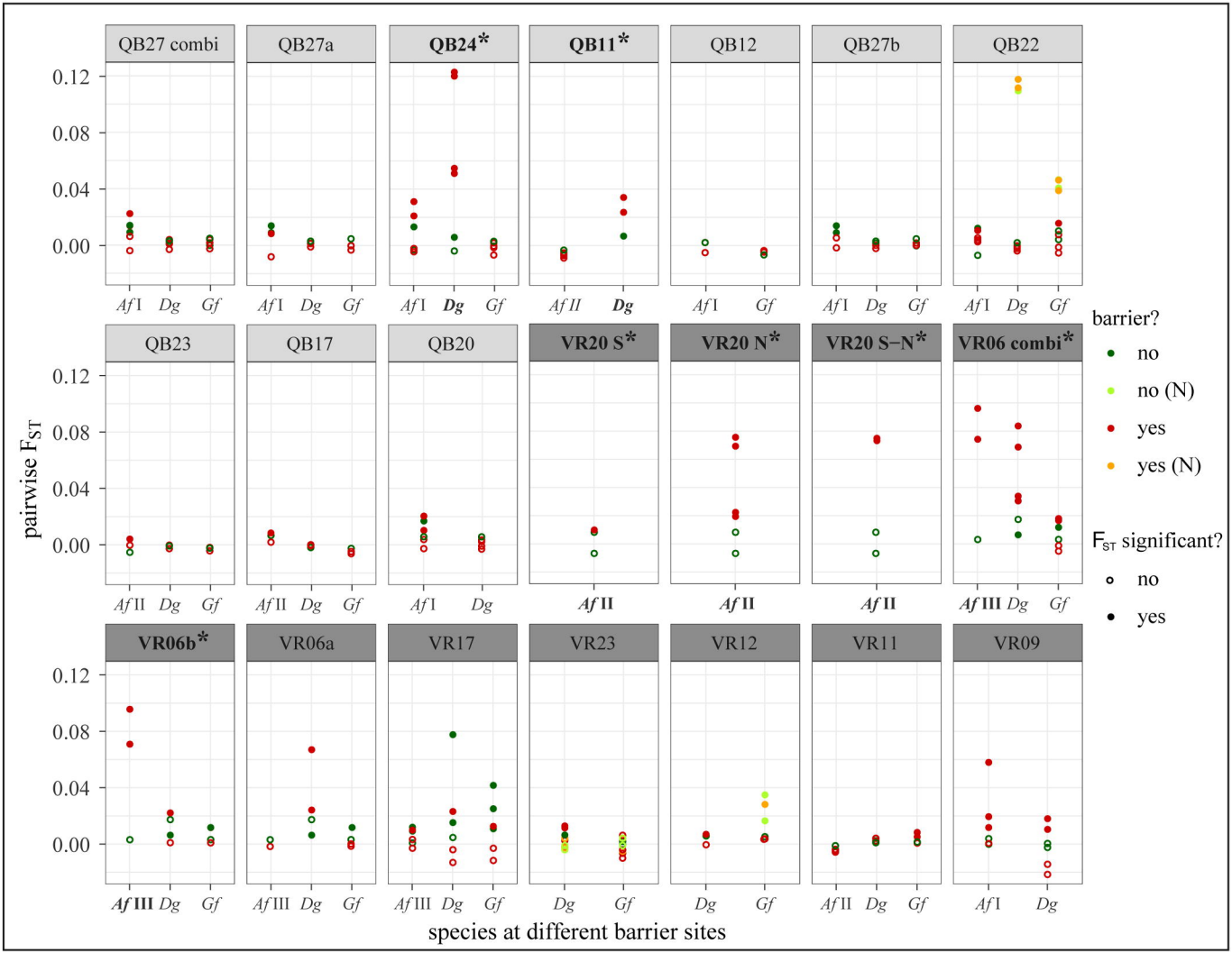
QB27 combi
QB27a
QB24*
QB11*
QB12
QB27b
QB22
QB23
QB17
QB20
VR20 S*
VR20 N*
VR20 S−N*
VR06 combi*
VR06b*
VR06a
VR17
VR23
VR12
VR11
VR09
pairwise F_{ST}
0.12
0.08
0.04
0.00
Af I
Af II
Af III
Dg
Gf
species at different barrier sites
barrier?
no
no (N)
yes
yes (N)
F_{ST} significant?
no
yes