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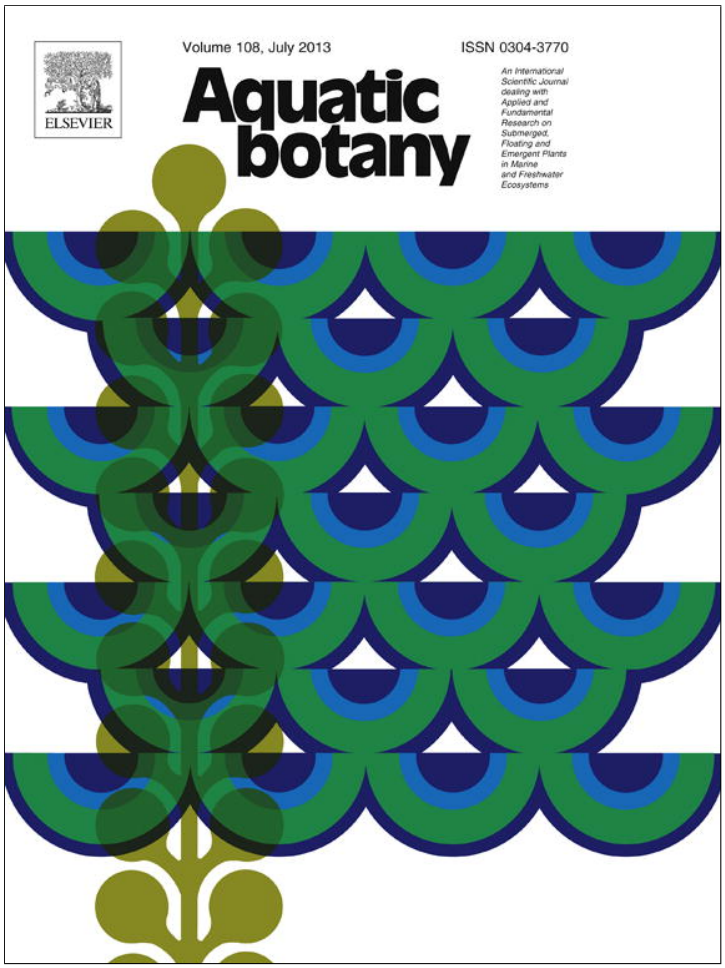

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Does water facilitate gene flow in spore-producing plants? Insights from the fine-scale genetic structure of the aquatic moss *Rhynchostegium riparioides* (Brachytheciaceae)

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ABSTRACT

Hydrochory plays a key role in the maintenance, diversity and evolution of aquatic plants and has traditionally been thought to (i) decrease or erase patterns of isolation-by-distance, (ii) increase outbreeding, and (iii) result in a downstream increase of genetic diversity. These hypotheses, which are especially relevant in organisms with water-mediated fertilization and a high ability to disperse vegetatively, are tested here from fine-scale spatial analyses of genetic variation at both the haploid and diploid phases in the aquatic moss *Rhynchostegium riparioides* (Hedw.) Cardot. A substantial geographical partitioning of genetic variation was found at the scale of the river basin and indirect measures of dispersal point to an overall weaker dispersal ability of the moss diaspores than pollen or wind-dispersed seeds. These observations, as well as the high F_{is} observed at the diploid stage and the very close proximity of potential fathers of the heterozygous diploid genotypes, strongly challenge the hypothesis that water enhances sperm and diaspore-mediated gene flow. In *R. riparioides*, the severe dispersal limitations revealed by the spatial analyses of genetic structure suggest that shoot fragments rather than spores are involved in local dispersal, which is fully consistent with the low levels of genetic diversity observed within populations. Extremely limited routine dispersal by unspecialized vegetative diaspores, coupled with discrete episodes of local population extinctions and colonizations by spores, account for the absence of a downstream increase of genetic diversity. As a result, although spore-producing plants are typically seen as most efficient dispersers, and although streams are traditionally seen as dispersal corridors, severe limitations of clonal dispersal at the local scale and discrete opportunities of random spore dispersal and establishment among colonies, both explain why typical metapopulation processes also apply to aquatic mosses.

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1. Introduction

Hydrochory, the passive dispersal of organisms by water, plays a key role in the maintenance, diversity and evolution of aquatic plants (Nilsson et al., 2010). Because it is considered to facilitate the downstream migration of diaspores (Pollux et al., 2009; Triest et al., 2010), hydrochory has traditionally been thought to enhance the spatial shuffling of genetic variation (Kudoh et al., 2006). As a result, low levels of genetic differentiation (see Nilsson et al., 2010 for review), and the erasure of any signal of isolation-by-distance (Ruckelshaus, 1998; Chen et al., 2007; Pollux et al., 2009), have been recurrently reported among populations of aquatic plants. The reduction of the spatial aggregation of close relatives due to the passive downstream dispersal of diaspores further likely promotes outbreeding (Kudoh et al., 2006). In fact, comparatively high levels of heterozygosity were found within populations of aquatic plants (Lloyd et al., 2011; Tanaka et al., 2011). Finally, the downstream migration of propagules theoretically results in a downstream gradient of genetic diversity (the unidirectional diversity hypothesis, see Honnay et al., 2010 for review).

An increasing number of population genetic studies have addressed these hypotheses, but their conclusions remain equivocal (Honnay et al., 2010; Nilsson et al., 2010). Honnay et al. (2009) suggested that the relationships between the direction and the distance of gene flow on the one hand, and measures of isolation by distance and population genetic differentiation on the other, may be considerably confounded by recurrent population extinctions and recolonizations. Genetic differentiation between populations may hence increase considerably in response to metapopulation dynamics and founder effects (Pannell and Charlesworth, 2000).

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In the present paper, we determine whether freshwater connectivity affects spatial patterns of genetic structure and diversity in aquatic mosses. Mosses disperse by means of spores, specialized vegetative propagules and gametophyte fragments. They require water for fertilization as a vestige from aquatic heritage. Although the process may be facilitated by micro-arthropods (Cronberg et al., 2006a), fertilization ranges are extremely limited. Male gamete dispersal distances range between a few centimeters (Rydgren et al., 2006) to a few meters (van der Velde et al., 2001). In terrestrial environments, the density of spores decreases with the distance from the mother gametophyte (Pohjamo et al., 2006). Trapping experiments suggest that a majority of spores disperses, however, beyond the nearest vicinity of the source. Regular establishment occurs at the km-scale (Lönnel et al., 2012), while 1% of the regional spore rain is assumed to have a trans- or intercontinental origin (Sundberg, 2012). Indirect estimates of migration derived from the analysis of the spatial genetic structure nevertheless remain controversial (Korpelainen et al., 2012). Some studies point to the significance of random founding effects by one or a few individuals followed by clonal growth (Cronberg et al., 2006b; Gunnarsson et al., 2007). Others demonstrate the crucial role of continuous establishment opportunities, giving rise to high genotypic diversity within sites (e.g. Cronberg, 1996, 2002; Stenøien and Sastad, 1999; van der Velde et al., 2001). While the viability of moss diaspores in water was previously investigated (Dalen and Söderström, 1999), no study has yet determined whether streams and rivers might indeed serve as dispersal corridors. Given the high potential for shoot fragmentation in bryophytes and the likelihood of subsequent passive downstream dispersal, Heino and Virtanen (2006) proposed that aquatic moss species are more widely distributed and show a stronger distribution-abundance relationship within a river basin than non-aquatic species.

From the fine-scale spatial analysis of genetic variation at both the haploid and diploid phases in the aquatic moss *Rhynchostegium riparioides* (Hedw.) Cardot, we assess whether growth within an aquatic environment enhances the likelihood of fertilization, and hence, rates of heterozygosity. We further evaluate the degree of population genetic differentiation and examine whether water connectivity reduces spatial aggregation of close relatives and hence erases any signal of isolation by distance at the scale of the river basin. Finally, we test the relevance of the 'unidirectional diversity hypothesis', which predicts increasing genetic diversity in downstream populations due to constant drift of alleles in a downstream direction.

2. Materials and methods

2.1. Sampling and molecular methods

R. riparioides is a robust monoicous pleurocarpous moss forming extensive mats covering submerged or occasionally emerged rocks in fast-flowing streams. It frequently and massively produces emergent sporophytes. It does not produce specialized gemmae, but whole gametophyte fragments can easily detach and disperse clonally. It was selected here as a model because (i) the species has been most recently recircumscribed taxonomically (Hustemékers et al., 2012); (ii) it is widespread across Europe, North Africa and western Asia, where it is locally dominant in running water communities and is hence used as a bioindicator (Ceschin et al., 2012; Manolaki and Papastergiadou, 2013); (iii) and specific nuclear microsatellite markers have been designed (Hutsemékers et al., 2008), facilitating the analysis of its genetic structure.

R. riparioides was sampled at 9 localities along a 12 km-long transect within the Lienne (Meuse hydrographic network, southern Belgium), a fast-flowing stream with an average oxygen saturation of 95% and an average annual discharge of 2.9 $m^3 s^{-1}$ at the junction with the Ambleve river (Fig. 1). Within each locality, 5–36 specimens were collected every meter along a 5–40 m long transect (Table 1). Altogether, 156 gametophytes (haploid stage) were sampled. All the populations but 2 and 5 included fertile specimens. Our sampling included 119 fertile gametophytes. All of the sporophytes (diploid stage, 1–6 per gametophyte) found on those gametophytes were sampled for a total of 169 sporophytes. Individual gametophytes and sporophytes were genotyped at six variable nuclear microsatellite loci following the protocols of Hutsemékers et al. (2008). Since the observed frequency of individuals with identical genotypes was systematically higher than the probability that two individuals from a theroretical non-clonal population exhibit the same multilocus genotype by chance (homoplastic mutations) (Hutsemékers et al., 2010), this protocol allowed to describe the spatial clonal structure of the populations.

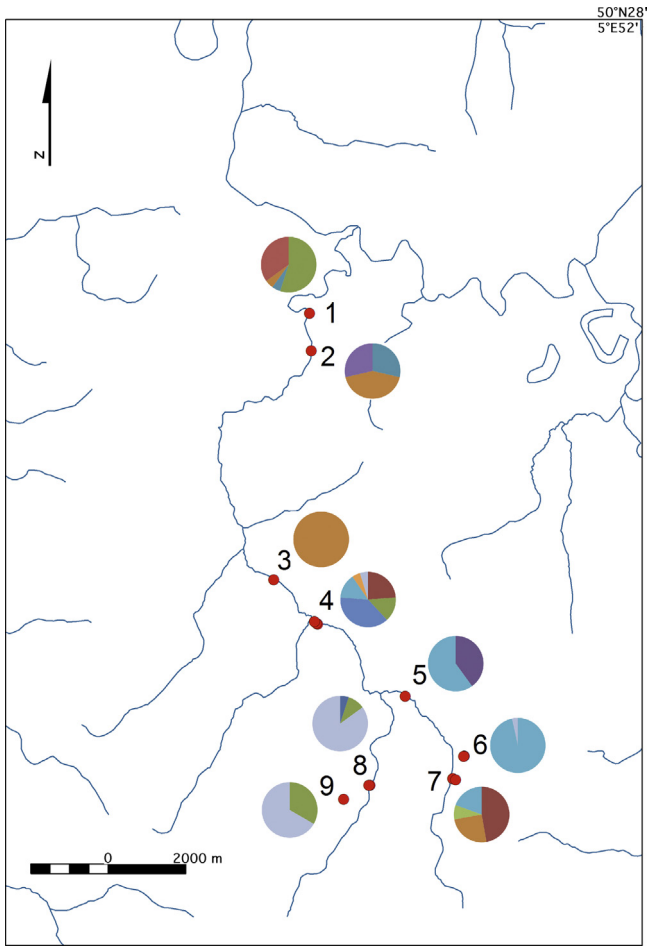

Fig. 1. Sampling design of the aquatic moss *R. riparioides* within the Lienne hydrographic network (southern Belgium). Pie diagrams represent the multilocus genotypic frequencies at six SSR markers within each of the 9 sampled localities.

2.2. Statistical analyses

Uni- and multilocus Wright's fixation indices (F_{IS}) were computed for the diploid sporophytic populations to analyze the mating system. Assuming the absence of selection and null alleles, F_{IS} provides an estimate of the inbreeding coefficient and can be used to infer an amalgam of both intergametophytic (among individuals from different spores within the same sporophyte) and intragametophytic (among individuals from the same spore after clonal fragmentation of the protonema) selfing in

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Table 1
Genetic diversity of 9 populations of the moss *R. riparioides* in the Lienne river (southern Belgium). *N*, sampling size; N_g, number of genotypes (number of private genotypes). *Ar*, allelic richness (corrected for sample size); *G*, gene diversity (corrected for sample size).

Population	1	2	3	4	5	6	7	8	9
N	20	14	5	21	5	29	36	20	6
N_g	4 (1)	3 (1)	1 (0)	6 (2)	2 (1)	2 (0)	4 (1)	3 (1)	2 (0)
N_g/N	0.20	0.21	0.20	0.29	0.40	0.07	0.11	0.15	0.33
Ar	1.47	1.62	1.00	1.49	1.49	1.07	1.65	1.21	1.16
G	0.214	0.295	0.000	0.232	0.294	0.023	0.272	0.080	0.089

haploid-dominant species with combined sexes (Eppley et al., 2007). The standard error of the multilocus F_{IS} was computed by jackknife over loci with Spagedi 1.3 (Hardy and Vekemans, 2002).

The genetic diversity of each haploid population (i.e. gametophytes only) was expressed in terms of unbiased expected heterozygosity (*He*) and allelic richness corrected for sample size (*Ar*) with GenAlex (Peakall and Smouse, 2006) and Fstat 2.9.3 (Goudet, 1995). To test the hypothesis of a downstream gradient of diversity, variation of *He* and *Ar* was correlated with geographic distance along the river from the downstream population 1 (Fig. 1).

The spatial genetic structure was examined by computing the global multilocus F_{ST} among populations. Significance of F_{ST} was assessed by 999 random permutations of individuals among populations and its standard error was obtained by jackknife over loci as implemented by Spagedi 1.3. We further investigated variation in pairwise kinship coefficients (F_{ij}) between gametophytes along gradients of geographic distance. In order to investigate whether dispersal primarily occurs by hydrochory or anemochory (i.e. dispersed by wind), two types of distance matrices were computed: one recording the nearest aerial distance among sites, the other recording pairwise distances following the river course from upstream to downstream populations, with other pairwise distances coded as missing. The significance of the slope of the regression of F_{ij} on the logarithm of the spatial distance between individuals was tested by means of 999 random permutations of population locations (Mantel test) with Spagedi 1.3. Mean F_{ij} values were also plotted against a gradient of predefined geographic distance intervals ranging between 0 and 12,000 m, the first interval corresponding to pairs of individuals from the same population. Significance of the mean F_{ij} per distance class was tested by 999 random permutations of individuals. The *Sp* statistics, a measure of the decrease of kinship coefficients between individuals with the logarithm of the distance separating them, was measured as $-\hat{b}/\left(1-\hat{F}_{(1)}\right)$, where $\hat{b}$ is the slope of the regression between all pairwise F_{ij} comparisons and the logarithm of the distance separating each pair of individual, and $\hat{F}_{(1)}$ is the mean F_{ij} among all pairs of neighbors (Vekemans and Hardy, 2004).

3. Results

Ten and twelve multilocus genotypes were found among the 156 gametophytes and 119 sporophytes, respectively. Wright's global fixation index was $F_{IS} = 0.95 \pm 0.02$. Within populations, $F_{IS} = 1.00 \pm 0.00$ at all the localities where sporophytes were sampled, with the exception of locality 7, where $F_{IS} = 0.90 \pm 0.01$. In fact, only 3 of the 169 diploid genotypes were heterozygous (one at two loci and two at three loci), and all were found at the same locality (7). Two paternal alleles were involved in those heterozygous combinations, and both of them were also found at the homozygous state in the same population. Table 1 summarizes the genetic diversity metrics of each of the 9 populations. Multilocus genotypes were found to be randomly distributed along the river course (Fig. 1).

Unbiased *He* in the haploid population ranged from 0.08 ± 0.04 to 0.295 ± 0.13 (Fig. 2). *He* and *Ar* were not significantly correlated to geographic distance along the river from locality 1 ($r = -0.35$ and -0.36, respectively, $p > 0.05$).

The global multilocus F_{ST} among populations was 0.43 ± 0.05 ($p < 0.001$). F_{ij} values quickly decreased along a gradient of geographic distance and the Mantel test between pairwise F_{ij} and geographic distance revealed a significant signal of isolation-by-distance, both employing shortest linear distances ($r = -0.19$, $p = 0 < 0.01$) and distances following the river course ($r = -0.27$, $p < 0.001$) as predictors (Fig. 3). The slope of the regression between all pairwise F_{ij} comparisons and the logarithm of the distance separating each pair of individual following the river course was -0.019. $\hat{F}_{(1)}$ equaled to 0.33, and the *Sp* statistics was 0.029.

4. Discussion

4.1. Selfing and clonal patterns at the local scale

The global multilocus F_{IS} observed in the sporophytic phase ($F_{IS} = 0.95$) lies within the upper bound of F_{IS} values reported for

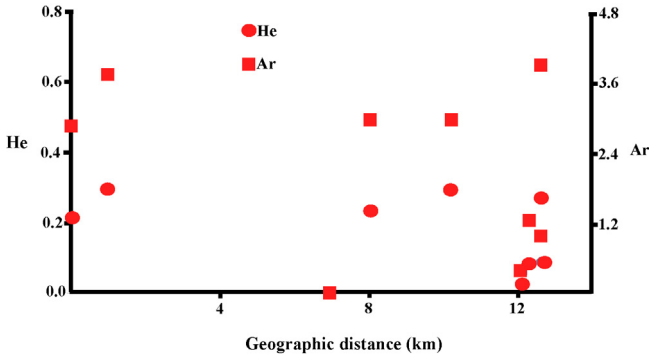

Fig. 2. Expected heterozygosity *He* and allelic richness *Ar* within populations of the aquatic moss *R. riparioides* sampled at 9 localities along the Lienne river (southern Belgium) along a gradient of aerial geographic distances from Locality 1 (see Fig. 1).

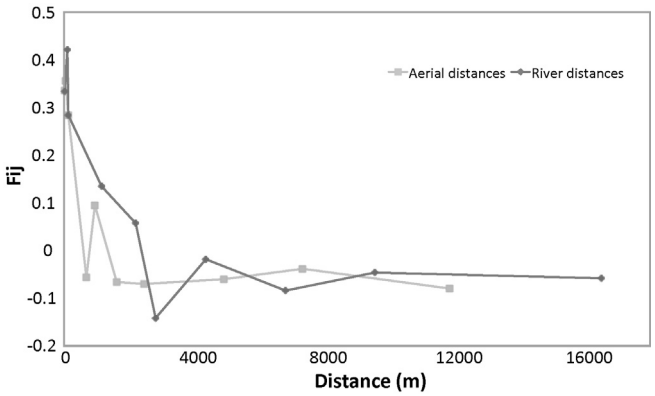

Fig. 3. Mean (and standard deviation bars) kinship coefficients F_{ij} between individuals for a set of spatial distance intervals in the aquatic moss *R. riparioides* within the Lienne hydrographic network (southern Belgium), considering aerial or river distances (see text for details).

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monoicous mosses (0.62–0.98, Eppley et al., 2007). This confirms the notion that selfing occurs frequently in monoicous moss populations (Eppley et al., 2007). Population genetics theory predicts that selfing may be selected against because it increases levels of homozygosity in offspring, which in turn, allows the expression of recessive, deleterious alleles (Charlesworth and Charlesworth, 1998). While outcrossing and, in particular, polyandry (Szovenyi et al., 2009) are selected for in dioicous mosses to avoid sporophyte inbreeding depression, recessive deleterious mutations are, in contrast, rapidly purged in autoicous mosses through intragametophytic selfing (Taylor et al., 2007). Once deleterious alleles are purged from the genome, selfing can be adaptive and general surveys in angiosperms confirm that complete selfing commonly occurs (Barrett and Eckert, 1990). Complete selfing as observed in all but one of the populations surveyed here might hence play a key role in maintaining local adaptations along environmental clines because of the large hitchhiking effects expected under high selfing (Vekemans and Hardy, 2004). In fact, the significant correlation found between genetic and ecological variation in *R. riparioides* at the regional scale is suggestive of reproductive isolation among ecotypes (Hutsemékers et al., 2010).

4.2. Dispersal limitations

At the haploid level, a substantial geographical partitioning of genetic variation was found at the scale of the Lienne river basin. Thus the F_{ST} among haploid gametophyte populations of *R. riparioides* (0.43) is significant and comparable to the range of values reported for terrestrial monoicous moss species at the regional scale (0.36–0.78, Eppley et al., 2007). The *Sp* statistics of *R. riparioides* in the Lienne basin (0.029) further demonstrates severe routine dispersal limitations. The *Sp* of *R. riparioides* indeed lies within the range of values reported for terrestrial angiosperm selfers (0.14 ± 0.08, Vekemans and Hardy, 2004). It is much higher than that reported for spermatophytes with wind pollination (0.006 ± 0.003), wind-dispersed seeds (0.01 ± 0.01) (Vekemans and Hardy, 2004), and even specialized vegetative gemmae in liverworts (0.01, Korpelainen et al., 2011). These observations, as well as the high F_{IS} observed at the diploid stage and the very close proximity of potential fathers of the three observed heterozygous diploid genotypes (in fact, within the same population), strongly challenge the hypothesis that water might enhance sperm and diaspore-mediated gene flow.

The fundamental question of how far hydrochores can be dispersed in a river and where they are finally deposited and germinate depends on a variety of intrinsic and extrinsic factors (Nilsson et al., 2010). Extrinsic factors include channel size, boundary conditions and hydraulic roughness, and morphology of the river, which determine flow hydraulics at different times during the season. The Lienne river is, however, a typical fast-flowing mountain stream whose hydraulic characteristics do not hamper downstream migrations.

Intrinsic factors include attributes of the seed, fruit, capsule, or vegetative propagule itself such as size, shape, floating ability, longevity, and other inherent factors that may fundamentally constrain or produce opportunities for long-distance dispersal (Nilsson et al., 2010). In *R. riparioides*, the severe dispersal limitations revealed by the spatial analyses of genetic structure suggest that shoot fragments rather than spores are involved in local dispersal. The shape of the relation between kinship coefficients and geographic distance is most similar to that observed in moss species reproducing asexually (Snäll et al., 2004). In addition, although shortest aerial distances and linear distances along the water course are significantly correlated, the matrix of linear distances following the water course accounts for a significantly higher percent variation of genetic variation than the matrix of shortest aerial distances, as shown by the differences in correlation coefficients with the two types of distances. Clonal fragmentation of shoot fragments is fully consistent with the low levels of genetic diversity observed within populations, with *He* values ranging from 0.08 to 0.295. These values, which fit with those reported for nuclear microsatellites or ISSRs in other clonally dispersed macrophytes (e.g., in *Potamogeton* and *Ottelia*, wherein $He < 0.2$, Chen et al., 2008, 2009), are in fact substantially lower than those reported for populations of outbreeding aquatic macrophytes, where in the range of *He* was for example 0.44–0.62 and 0.58–0.65 among populations of *Vallisneria* and *Zoostera*, respectively (Lloyd et al., 2011, Tanaka et al., 2011).

Since diaspore size is a factor of prime importance for dispersal ability in the case of anemochory (Pohjamo et al., 2006), a straightforward interpretation of the poor dispersal ability of *R. riparioides* at the basin scale would be that large shoot fragments exhibit low dispersal abilities. While such an interpretation potentially holds in a terrestrial environment and might explain, for instance, the unexpected low dispersal ability of obligate epiphytic bryophytes (Devos et al., 2011), dispersal distance is unrelated to diaspore mass in water (Ikeda and Itoh, 2001).

4.3. Relative importance of hydrochory and anemochory

Species with predominantly self-fertilizing and vegetative clonal reproduction usually display a higher percentage of genetic variation among populations than outcrossing species (Black-Samuelsson and Andersson, 1997; Gaudeul et al., 2000), suggesting more limited gene flow among populations. This alone may, however, not explain the strong geographic patterns of genetic variation and low migration ability of *Rhynchostegium*'s shoot fragments. In an experimental study in fact, Suren et al. (2000) demonstrated that populations of aquatic mosses submitted to a range of streamflow intensities lose 10–12% of biomass after the flood, resulting in the release of a substantial number of shoot fragments. Shoot fragments may be better suited for routine dispersal within the river basin than anemochorous spores. Indeed, asexual propagules allow for early reproduction, are less sensitive to habitat quality (Löbel, 2009), result in the production of new gametophytes at a faster rate (Mishler and Newton, 1988) and increase establishment frequency as compared to spores (Löbel et al., 2009; Löbel and Rydin, 2010).

In typical hydrochorous angiosperms, fruits and seeds display morphological and anatomical adaptations, such as air-filled tissue, which enable them to float for long periods of time, and other traits, such as dormancy, which enhance long-distance dispersal. Trapped air, corky tissue, and high surface area to volume ratios may be seen as exaptations enhancing hydrochorous dispersal (see Nilsson et al., 2010 for review). The shoots of *R. riparioides*, in contrast, do not exhibit any specific features that would enhance flotation. Seeds with low buoyancy are very likely to germinate underwater and develop a new plant. This is, however, not possible in the case of aquatic mosses, which display a narrow ecological niche regarding substrate quality (Glime and Vitt, 1987; Muotka and Virtanen, 1995; Hylander and Dynesius, 2006) and require appropriate rock surfaces to establish and resume growth. Hence, the results presented here suggest that, in the absence of any morphological adaptation, shoot fragments of *R. riparioides* tend to sink after a few tens to hundreds of meters, especially in tranquil river reaches where they cannot find the appropriate habitat.

This raises the question, however, why aquatic mosses fail to develop adaptations to flotation if, as the patterns of isolation-by-distance observed in *R. riparioides* unambiguously suggest, hydrochory is indeed the main dispersal strategy. Although the relatively low number of populations sampled may have prevented a statistically significant downstream increase in genetic diversity to emerge, the pattern of genotype diversity along the river course (Fig. 1) contrasts, however, with the idea of a one-way dispersal

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of diaspores and does not fit with the expectations of the unidirectional diversity hypothesis. Like in many other aquatic mosses in fact, the sporophyte structure of *R. riparioides* is identical to that of many terrestrial species, and spores are assumed to disperse by air (Vitt and Glime, 1984). The random genetic structure observed at the scale of the hundred of meters can hence be interpreted in terms of discrete episodes of local population extinctions and upstream migrations by spores. This interpretation fits with previous observations on the genetic structure of clonal mosses, wherein spatial patterns of genetic variation are explained by clonal reproduction within populations, and structured by sexual reproduction among populations (Cronberg et al., 2006b). A clearer downstream increase in genetic diversity could hence be expected in purely clonal species. The absence of any relationship between genetic similarity and distance after a few hundreds of meters in sterile aquatic mosses suggests, however, that rare events of upstream dispersal of gametophyte fragments by waterfowls followed by clonal propagation may also take place (Korpelainen et al., in press). As a result, although spore-producing plants are typically seen as most efficient dispersers (see Hutsemékers et al., 2011 for review) and although streams are traditionally seen as dispersal corridors, severe limitations of clonal dispersal at the local scale, and discrete opportunities of random diaspore dispersal and establishment among colonies, both explain why typical metapopulation processes also apply to aquatic mosses.

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