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Genetic assessment of *Luronium natans* in lower Belgium

Analysis of population connectivity in an aquatic perennial

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Summary

Luronium natans (L.) Raf. or Floating Water-plantain, an aquatic perennial, is endemic to west and central Europe. In Belgium, the number of sites with *L. natans* decreased markedly in the 20th century. The species is considered "vulnerable" on the national Red List and its conservation status as "unfavourable". Considering its mixed reproduction strategy and polyploidy, population genetics could provide insight into its genetic vulnerability. We investigated genetic variation within and among 30 locations from various habitats in Flanders using amplified fragment length polymorphic markers. Besides information on clonality and genetic diversity, we tried to include information on (former) population connectivity.

We found a high degree of clonal reproduction in the investigated locations. Mostly, a dominant multilocus lineage (clone) was shared among patches within a water body. But also an occasional clone was found among distant locations. Nevertheless, populations of *L. natans* were highly differentiated, but proved to be generally low in genetic diversity. It appears several populations were founded by one or a few migrants from neighbouring populations and were able to persist mainly through asexual reproduction. Although census population size and genetic diversity are related, clonal reproduction confounds this trend. Considering the strong population dynamics, periodical evaluation of the within-population genetic diversity is necessary to gain insight in the importance of sexual reproduction and seed bank recruitment.

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1 Introduction

Luronium natans (L.) Raf. or Floating Water-plantain is an aquatic perennial of the Alismataceae, and is endemic to west and central Europe. It is a stoloniferous pioneer with a low competitive ability, but its fundamental niche is rather broad (Greulich et al., 2000b; Szańkowski & Kłosowski, 2001; Vanderhaeghe et al., 2013; Willby & Eaton, 1993). *L. natans* grows in weakly to well buffered oligotrophic and mesotrophic shallow waters, in the littoral zone of deep lakes, as well as in more nutrient rich streams (Kay et al., 1999). Depending on the type of habitat, its phenotype varies considerably (Kay et al., 1999): 1) rosettes with short, linear-lanceolate leaves (isoetid), such as in deeper parts of carbon-poor lakes, 2) isoetid with long leaves, in habitats richer in carbon dioxide and deeper parts of flowing water, 3) nymphaeid with submersed linear leaves and floating leaves in shallow parts of fens, pools, lakes and slow flowing rivers, 4) with only short stemmed lanceolate-ovate leaves on semi-terrestrial locations, such as in temporary ponds and on shores.

Luronium natans occurs from southern Norway and Sweden in the north, through Ireland and France to northern Spain, east to Poland and the Czech Republic, but was always rare outside the western part of its range. The species is listed on Annex II of the EU-Habitats Directive and under Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention), but is considered to be of "least concern" on the IUCN Red List of Threatened Species (Lansdown, 2011). Although the number of locations with *L. natans* is reported to decline rapidly (Cook, 1983), Lansdown & Wade (2003) indicate that available records are often inadequate to assess status accurately, generally because of difficult identification (e.g. Romero et al., 2004), the trouble of locating populations, limited surveys and the dynamic response of metapopulations to natural and anthropogenic environmental changes. Besides habitat destruction, acidification and eutrophication contributed most to the demise of the species in its principal area of occurrence. In Belgium, the number of sites with *L. natans* is reported to have decreased markedly in the 20th century (Ronse, 2006). Although recent inventories indicated that the species was sometimes overlooked (Ronse et al., submitted). Also, (re)establishment at new sites occurs, often as a result of restoration measures. Nevertheless, *L. natans* is considered as "vulnerable" on the national Red List (Kestemont, 2010) and its conservation status as "unfavourable" (Louette et al., 2013).

We studied *L. natans* in lower Belgium (Flanders), where it used to be fairly common in the Kempen region, north of the rivers Demer and lower Dijle. Some isolated populations were also present more to the west, in sandy Flanders up to the year 2000, but these seem to have disappeared. Evaluation of the local conservation status is based on observations of population structure, size and reproductive traits, as well as of certain biotic and abiotic conditions that characterize habitat quality (Denys et al., 2008). For now, the genetic variation of populations was not considered to assess local conservation status. Positive relationships exist between population size, plant fitness and within-population genetic diversity, especially in self-incompatible species and to some extent in rare species (Leimu et al., 2006). *L. natans* can, however, reproduce vegetatively as well as sexually, which can complicate the estimation of genet population size. Counting the number of ramets will not result in a reliable estimate of the number of genets and consequently of the effective population size (Tepedino, 2012). Overestimation of population size seems therefore plausible. Still, clonal populations do not necessarily display low fitness (e.g. De Witte et al., 2012), as occasional sexual reproduction, migrants and mutations can reduce possible negative effects (Ellstrand & Roose, 1987; Widén et al., 1994). Also, clonality can ensure population persistence (de Witte & Stocklin, 2010). Furthermore, *L. natans* might be considered self-compatible in the form of cleistogamy (Kay et al., 1999; Lansdown & Wade, 2003; Sculthorpe, 1967), reducing the possible problem of mate or pollen limitation that comes with clonal reproduction for obligatory outcrossing species (Honnay & Jacquemyn, 2008). Although submerged flowers of *L. natans* may set seed through selfing, it remains unknown to what extent these produce viable offspring. As *L. natans* is hexaploid, high levels

of genetic diversity might still be present that could counterbalance genetic erosion due to genetic drift or loss of genets in relic populations (Brochmann et al., 2004). Polyploidy and clonality often co-occur in plants (Brochmann et al., 2004; Gustafsson, 1948). Contrary to diploids, polyploids are able to compensate for high mutational loads by having more than two copies of each locus and thereby display higher average heterozygosity levels, making them less prone to inbreeding depression caused by geitonogamy (Eckert, 2000; Husband et al., 2008).

Considering its mixed reproduction strategy and polyploidy, population genetics could provide insight into its genetic vulnerability. So far, genetic studies on *L. natans* were done using isozymes on populations in Wales and Ireland (Kay et al., 1999) and using amplified fragment length polymorphic markers (AFLP) on Danish river and lake populations (Nielsen et al., 2006). Both studies investigated genetic variation within and among (sub)populations. We performed a similar analysis of 30 locations from various habitats in Flanders using AFLP. Besides information on clonality and genetic diversity, we tried to include information on (former) population connectivity. Results are discussed in relation to suggested criteria for the evaluation of local conservation status.

2 Material and methods

2.1 Sampling and population characteristics

Leaves were collected in 2009-2010 in most known locations with *Luronium natans* following a strict sampling scheme and stored on silica gel. Floating (also called "expanded leaves"; Lansdown & Wade, 2003) or terrestrial leaves were preferred for sampling; in a few cases, submerged, linear leaves were sampled. Total sample size was restricted by the responsible government agency, the Flemish Agency for Nature and Forest, and depended on the conservation status of the concerning population. Where conservation status was considered favourable (the total area > 5 m², number of ramets > 100, presence of flowering/fruiting individuals; Denys et al., 2008) a maximum of 30 individuals were sampled.

We intended to study the clonal reproduction within a patch using a particular sampling design. Where more than six patches of *Luronium natans* occurred, a linear transect of 4.4 m was outlined along the maximum diameter of the largest one. Plants were sampled 40 ± 10 cm apart, starting from the edge of the transect (Figure 1). When the largest patch was less than 4.4 m long, an additional transect was sampled in the second largest one, until twelve plants were included. Six patches were randomly selected. If the largest patch was included in this selection, three samples were added to the linear transect with a maximum of fifteen samples. Three plants were sampled from the other selected patches, with one in the middle of the patch and 1 m between individuals, where possible. For (sub)populations with a good conservation status but less than six patches, three samples were added to the transect in the largest patch (with a total of fifteen samples). Fifteen additional plants were sampled from all other patches.

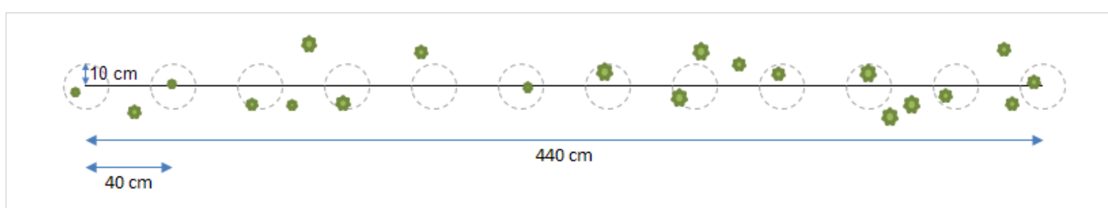

Figure 1 Method of sampling *Luronium natans* in a linear transect. There are 12 sampling points along a 4.4m transect line. A sample is taken from a plant (green stars) if it is within 10 cm of a sampling point.

In a few cases, more than fifteen samples were collected in the transect to achieve the goal of 30 samples in total. Still, a sample size of 30 could not be realized in every population with a good conservation status.

In case of an unfavourable conservation status, the number of samples was restricted to 10. Preferentially, these were taken from a linear transect in the biggest patch, as described above. If this transect had less than ten individuals, further samples were again divided over the other patches. If only isolated plants were present, up to ten individuals were sampled.

Table 1 lists all locations studied (see also Figure 4 for their location). Coordinates of all patches were acquired by GPS. The following population characteristics were estimated: census population size (ordinal with six levels: <10, 10 to 19, 20 to 49, 50 to 99, 100 to 999 and >1000 ramets), presence of flowers (binary) and history (Table 1).

Table 1 List of sampling locations of *Luronium natans* visited in 2009 and 2010. ID: population ID; AFLP samples: number of samples successfully analysed with AFLP.

location	**ID**	**year**	**number of patches sampled**	**plants sampled**	**AFLP samples**	**total number of patches**	**population size**	**flowers present**	**river basin**	**type**	**history**
Bergerven	BEVE	2009	4	21	20	4	100-1000	yes	Maas/ Meuse	pit	older relic
Daelemansloop	DAEL	2009	8	30	29	65	>1000	yes	Nete	stream	older relic
Fort Ertbrand	ERTB	2009	5	16	15	9	50-100	yes	Lower-Scheldt	canal	older relic
Goorvijver	GOVI	2009	1	2	2	1	2	no	Nete	pit	colonized (<50 yrs)
Groene Delle	GRDE	2009		1	0						
Grote Dorst	GRDO	2009	4	10	10	4	50-100	yes	Demer	pond	older relic
Kapellen Heidestraat Noord	KAPEL	2009	1	24	19	1	50-100	yes	Lower-Scheldt	canal	older relic
Kleinebeek	KLBE	2009	2	2	2	2	3	no	Demer	stream	older relic
Mellevijver	MELLE	2009	6	16	14	6	50-100	yes	Nete	pit	colonized (<50 yrs)
Volharding Gracht	RIJK	2009	1	6	3	1	ca. 20	no	Nete	pit	colonized (<50 yrs)
Roskampsputje	ROSK	2009	4	30	25	4	100-1000	yes	Demer	pond	older relic
Slaaphuis	SLAA	2009	3	16	14	3	100-1000	yes	Maas/ Meuse	ditch	older relic
Tenhaagdoornheide noord	TENHN	2009	2	2	2	2	1-10	no	Demer	pond	older relic
Tenhaagdoornheide zuid	TENHZ	2009	3	10	8	3	20-50	Yes	Demer	pond	older relic
Vorsdonkbos	VORS	2009	1	5	5	1	12	no	Demer	pond	older relic
Welleken	WELL	2009	1	10	8	1	20-50	yes	Demer	pond	restored
Wik nr. 1k	WIK1k	2009	5	10	9	5	50-100	yes	Demer	pond	older relic

location	ID	year	number of patches sampled	plants sampled	AFLP samples	total number of patches	population size	flowers present	river basin	type	history
Wik nr. 1	WIK1	2009	7	10	7	>20	>1000	yes	Demer	pond	older relic
Wik nr. 11	WIK11	2009	5	10	7	5	50-100	yes	Demer	pond	older relic
Wik nr. 12	WIK12	2009	floating plants	10	8	0	10-20	no	Demer	pond	older relic
Wik nr. 13	WIK13	2009	1	2	1	1	1-10	no	Demer	pond	older relic
Wik nr. 3	WIK3	2009	8	10	10	10	10-20	yes	Demer	pond	older relic
Wik nr. 7	WIK7	2009	floating plants	10	8	0	50-100	no	Demer	pond	older relic
Wik nr. 8	WIK8	2009		1	1						older relic
Zwart Water	ZWWA	2009	1	6	5	1	20-50	yes	Nete	pond	older relic
Bosvijver	BOSV	2010	8	30	29	≥ 8	100-1000	yes	Demer	pond	older relic
Langdonken 1	LANG1	2010	8	30	28	≥ 8	100-1000	yes	Nete	ditch	restored
Langdonken 2	LANG2	2010	2	2	2	2	1-10	yes	Nete	ditch	restored
Goorplasje	GOPL	2010	1	1	1	1	1	no	Nete	pond	restored
Volharding Plas	RIJKP	2010	17	29	26	NA	100-1000	no	Nete	lake/pond	colonized (<50 yrs)
Dauteweyers	DAUT	2010	9	21	19	9	100-1000	yes	Demer	lake/pond	restored
Doodsgeleeg	DOOD	2010	9	30	30	≥ 9	100-1000	yes	Demer	pond	older relic

2.2 DNA extraction and AFLP analysis

Total DNA was extracted from ground leaf samples with QuickPick™ SML Plant DNA purification kit in combination with the PickPen 8-M magnetic tool (Isogen Life Science). The integrity and quantity of DNA were assessed on 1.5% agarose gels and spectrophotometrically with the ND-1000 Nano-Drop (NanoDrop Technologies), respectively. AFLP DNA fingerprints were generated according to Vos et al. (1995), but with restriction and ligation conducted in one single step. After testing 32 primer combinations, six were retained: PstI-ACG/MseI-CAC (PC1), PstI-ACG/MseI-CCG (PC2), PstI-ACG/MseI-CCA (PC3), PstI-ACG/MseI-GAT (PC4), PstI-ACT/MseI-CTC (PC5), PstI-ACT/MseI-GCT (PC6). Fragment separation and detection was performed on a NEN IR2 DNA analyser (LI-COR Biosciences) using 36 cm denaturing gels with 6.5% polyacrylamide. IRDye size standards (50–700 bp) were included for sizing of the fragments. All samples were run in a randomized fashion. AFLP banding patterns were scored using SAGAMX software (LI-COR Biosciences). For the analysis of samples collected in 2010, we included 32 samples from 2009 to enable comparison of both sets. To test for reproducibility, 20 samples (7.4% of the 270 samples) from 2009 and 37 (21.1% of 175 samples) from 2010 were blindly replicated within and between gels.

2.3 Data analysis

2.3.1 Clonality

Identification of clones from genotypic information may be affected by genotyping errors. When molecular markers are insufficiently polymorphic, sibling fingerprints appear identical (Arnaud-Haond et al., 2005; Douhovnikoff & Dodd, 2003). In reverse, somatic polymorphisms (caused by somatic mutations) can lead to underestimation of the true degree of clonality. To evaluate the resolution of the markers we assessed if the genotypic resolution followed an asymptotic trend (Arnaud-Haond et al., 2005). We followed the methodology described by Cox et al. (2014).

Ramets (or clone mates) belonging to the same clonal lineage bear the same multilocus genotype or, if somatic mutations have occurred, the same multilocus lineage (MLL). To infer clonal identity we used GenoType (Meirmans & Van Tienderen, 2004). The frequency distribution of pairwise distances, in this case Dice similarities, can help reveal somatic mutations and genotyping errors (Arnaud-Haond et al., 2007). The distribution of pairwise distances in both datasets of 2009 and 2010 was more or less unimodal. Only for 2010 a slightly bimodal pattern emerged, with a very small primary peak for similarities above 0.98. Because this was comparable to the mean Dice similarity of 0.98 calculated for duplicate samples of 2009 and 2010, respectively, it was considered as the threshold to define MLLs. Clonal identity was calculated for each dataset separately, as the combined set, including only 81 markers, presented a higher error rate.

Relative population genotypic richness (*G/N*) was quantified as the number of unique MLLs divided by the number of samples. The following indices were computed for each population using GenClone 2.0 (Arnaud-Haond & Belkhir, 2007): the adapted Shannon index for clonal diversity, *H″*, the corresponding evenness index describing clonal equitability, *V′H″* and the adapted Simpson index for genotypic diversity, *D**, which decribes the chance of randomly taking two distinct MLLs in a sample (Arnaud-Haond et al., 2007). Because the number of loci to be used in GenClone is limited to 42, the MLL number was entered as a sole allele (Vandepitte et al., 2009). Minimum and maximum distances between the ramets of a MLL were calculated from the coordinates of the patches and average distances between samples within a patch, except for locations with missing coordinates of sampled patches.

2.3.2 Genetic diversity

Shannon's information index (*I*) and percentage of polymorphic loci (*PPL*) were calculated with Popgene v. 1.32 (Yeh et al., 1997) according to Lewontin (1972), with and without

possible clones. Initially Hardy-Weinberg equilibrium (F_{IS} = 0) was assumed but some inbreeding (F_{IS} = 0.1) was accounted for in final calculations. Spearman rank correlation between *I*, retaining unique ramets, and population size was calculated in R version 2.15.3 (R Development Core Team, 2010), WIK7 and WIK12 (Table 1) not included, where only free-floating plants were found.

2.3.3 Genetic structure

Analysis of Molecular Variance (AMOVA) in Genalex 6.4 (Peakall & Smouse, 2006; Peakall & Smouse, 2012) provided a general Φ_{PT} value, a measure of population differentiation, as well as its significance level (Monte Carlo procedure with 999 permutations). Computations were done for each separate dataset (of 2009 and 2010) as well as for the combined data. Furthermore, we performed a Principal Coordinate Analysis (PCoA) based on pairwise Nei's genetic distances (Nei, 1972) for individuals, and Nei's unbiased genetic distances (Nei, 1978) for locations. We used the covariance matrix with data standardization on each separate dataset and on the combined dataset.

Because most sampled (sub)populations are small and *Luronium natans* often reproduces vegetatively, Hardy-Weinberg may be violated. We therefore used non-hierarchical K-means clustering (Hartigan & Wong, 1979) with KMeans v. 1.1 (Meirmans, 2012) to detect a genetic structure. This assigns individuals to K genetic groups maximizing among-group variance (also intergroup inertia; Legendre & Legendre, 1998). Simulated annealing was conducted with 100,000 steps and 500 repeats of the algorithm. Both the pseudo-F statistic (Caliński & Harabasz, 1974) and the Bayesian Information Criterion (BIC; Schwarz, 1978) were evaluated to obtain the optimal number of clusters. The maximum number of cluster (K) was set to 40 for the clustering of individuals and patches. When locations were clustered, the number of locations could not be chosen as K, but only half the number of locations (restriction of the program).

We evaluated spatial patterns with a spatial component analysis (sPCA; Jombart et al., 2008) which is independent from Hardy-Weinberg assumptions or linkage disequilibrium. This multivariate method uses allele frequencies and accounts for their genetic variability and spatial autocorrelation, calculated with Moran's *I* (Moran, 1948; Moran, 1950). Because of the aggregated spatial distribution of the samples, we used a distance-based connection network, the neighbourhood by distance graph, with a minimum distance d_1 of 0 and a varying maximum distance d_2 of 0, 10 and 45 km. A d_2 = 0 suggests at least one connection in each patch (or population), the larger distances were deducted from the clonality results; the distance of locations sharing a MLL ranges from ca. 9 to 51 km. Smaller distances between clones can be found between well-connected water bodies. Global and local spatial structures were tested with 999 permutations and randomly distributed allele frequencies as the null hypothesis (Jombart et al., 2008). All calculations were done using the R package adegenet 1.3-6 (Jombart, 2008).

The relationship between pairwise geographic and pairwise Φ_{PT} values on the population level was assessed with a Mantel test using Genalex 6.4 with 999 permutations. Calculations were repeated for each dataset without potential clones. The geographic distances were first transformed as their base 10 logarithm plus one. We also performed a Mantel test for the locations of the Demer basin and Nete basin, respectively, using the combined dataset.

3 Results

3.1 AFLP pattern

Out of the 270 samples of 2009, 38 (14%) showed weak profiles for one or more primer combinations. This was also the case for twelve samples (7%) from 2010, including 4 reanalysed samples of 2009. These were excluded from further analysis (Table 1). The AFLP analysis of the samples from both years delivered 237 and 282 polymorphic markers, respectively. Mean typing error reached 2% for the samples of 2009 and 3% for the samples of 2010 per locus (following Bonin et al., 2004).

By comparing AFLP profiles of 2009 with the ones of 2010 for each primer combination, 81 common markers could be retrieved. Based on the 28 samples included in both datasets, the mean error rate was 8%.

Samples with deviating AFLP profiles were also outliers in a PCoA (results not shown). These possibly represented misidentifications or laboratory errors and were removed from analysis (WIK8-1-1, WIK12-4, ROSK-2-2, LANG1-4-2, RIJKP-4-1 and DAUT-1-7 with the sample ID entailing "location ID-patch number-sample number").

3.2 Clonality and genetic diversity

The genotypic resolution of the marker sets for 2009 and 2010 followed an asymptotic trend (results not shown), allowing reliable detection of MLLs. Based on the Dice similarity of 0.98, only 249 unique MLLs remained out of 362 samples. However, the reference samples of 2009 included in the dataset 2010 appeared to be different MLLs based on markers of 2009, but ramets according to markers of 2010: 6 MLLs of BEVE were reduced to 3 MLLs, 7 of MELLE to 2, 6 of WELL to 4. The three and seven samples of WIK1 and WIK12, respectively, remained different MLLs.

Clonal growth was common. The genotypic richness and diversity indices are shown in Table 2. Population genotypic richness (*G/N*) ranged between 0.17 and 1 (mean = 0.78). The maximum value of 1 mainly occurred in locations with only one to five samples; exceptions were WIK7 and WIK12 with eight samples of free-floating plants each, GRDO (ten samples), LANG1 (27 samples) and DOOD (30 samples). Shannon diversity values (*H″*) were generally moderate (0.43-3.40; mean = 1.65). The eveness index (*V′H″*) ranged between 0.39 and 1, generally remaining quite high (with a mean value of 0.88). Nevertheless, when clones were present in a population, there usually appeared to be one dominant MLL (Appendix 1, Table A 1). The eveness index might be therefore influenced by rare MLLs, whereas the Simpson index was not (Arnaud-Haond et al., 2007). The latter was mostly slightly lower than the eveness index (0.22-1 with mean = 0.85). Distances between potential ramets of a MLL within locations ranged between 0.2 and 513 m.

Table 2 Genotypic richness and diversity indices for the sampled locations of *Luronium natans*. N: number of samples; MLL: number of multilocus lineages; *G/N*: genotypic richness; Min. dist.: minimum distance between ramets of a MLL; Max. dist: maximum distance between ramets of a MLL; *H″*: Shannon index for genotypic diversity; *V′H″*: evenness index; *D**: Simpson index; NA: not available; NR: not relevant; SD: standard deviation.

ID	**N**	**MLL**	***G/N***	**Min. dist. (m)**	**Max. dist. (m)**	***H″***	***V′H″***	***D****
BEVE	20	10	0.5	0.4	22	1.676890	0.728264	0.710526
DAEL	29	16	0.55	0.4	183	1.918777	0.727069	0.738916
ERTB	15	7	0.47	0.45	93	1.389715	0.714172	0.657143
GOVI	2	2	1	NR	NR	0.693147	1	1

ID	N	MLL	*G/N*	Min. dist. (m)	Max. dist. (m)	*H"*	*V'H"*	*D**
GRDO	10	10	1	NR	NR	2.302585	1	1
KAPEL	19	10	0.53	0.2	3	1.732552	0.752438	0.736842
KLBE	2	2	1	NR	NR	0.693147	1	1
MELLE	14	8	0.57	NA	NA	1.666102	0.801226	0.769231
RIJK	3	3	1	NR	NR	1.098612	1	1
ROSK	24	14	0.58	0.4	20	2.079018	0.787788	0.800725
SLAA	14	6	0.43	0.8	10	0.994354	0.617827	0.505495
TENHN	2	2	1	NR	NR	0.693147	1	1
TENHZ	8	4	0.5	0.3	12	1.073543	0.774397	0.642857
VORS	5	5	1	NR	NR	1.609438	1	1
WELL	8	7	0.87	4.5	4.5	1.906155	0.979570	0.964286
WIK1k	9	6	0.67	0.4	11	1.676988	0.935945	0.888889
WIK1	7	6	0.86	41	41	1.747868	0.975504	0.952381
WIK11	7	6	0.86	0.4	0.4	1.747868	0.975504	0.952381
WIK12	8	8	1	NR	NR	2.079442	1	1
WIK13	1	1	1	NR	NR			
WIK3	10	9	0.9	30.9	30.9	2.163956	0.984859	0.977778
WIK7	8	8	1	NR	NR	2.079442	1	1
ZWWA	5	3	0.6	0.45	NA	0.950271	0.864974	0.7
BOSV	29	13	0.45	0.4	31	1.706447	0.665294	0.665025
LANG1	27	27	1	NR	NR	3.295837	0.992007	0.994302
LANG2	2	2	1	NR	NR	0.693147	1	1
GOPL	1	1	1	NR	NR			
RIJKP	25	19	0.76	1	513	2.765155	0.939111	0.956667
DAUT	18	3	0.17	0.4	10	0.425848	0.387624	0.215686
DOOD	30	30	1	NR	NR	3.401197	1	1
mean			**0.777**			**1.652166**	**0.878699**	**0.851040**
SD			**0.247**			**0.750718**	**0.158209**	**0.194407**

Clones appeared to be distributed among separate patches as well as among locations (Appendix 1, Table A 1). The following locations shared a MLL: ERTB – KAPEL, MELLE – TENHZ – ZWWA, WIK1 – WIK1k – WIK3 – WIK11 – WIK13. No MLL was shared among locations in the dataset of 2010.

The values for genetic diversity are shown in Table 3. The indices did not change with F_{IS} set at 0.1 (results not shown). For the dataset without potential clones within locations, three loci became monomorphic and were excluded from further analysis. Because of unequal and generally low sample sizes, the values should be assessed with caution. In most cases, the values of I slightly increased when possible clones were excluded. Where the proportion of clonality was high, a higher increase of I was, however, detected (e.g. DAUT). Values of I without possible clones, ranged from 0.01 to 0.39 (mean = 0.11) and were generally low. Exceptions are WIK12 and WIK7 represented by free-floating individuals, and locations DOOD, LANG1 and RIJKP that have a sample size ≥ 20 (i.e. number of MLLs). However, a larger sample with a moderate number of MLLs does not imply higher genetic diversity, as shown by the values of I for DAEL, ROSK and BOSV. Nevertheless, a positive correlation

exists between I (only unique ramets) and population size (R = 0.538, p = 0.0097). As population size largely determined sample size, both are also correlated (R = 0.691, p = 0.0004), as are I and sample size (R = 0.489, p = 0.0209). There is no clear link between genetic diversity and population history (Tables 1 and 3).

Table 3 Shannon's information index with (I) or without possible clones (I excl. clones) and their respective standard deviation (SD). N: number of samples; MLL: number of multilocus lineages; *PPL*: proportion polymorphic loci retaining unique ramets.

ID	**N**	**MLL**	***I***	**SD *I***	***I* excl. clones**	**SD *I* excl. clones**	***PPL***
BEVE	20	10	0.0489	0.1346	0.0691	0.1615	16.67%
DAEL	29	16	0.1091	0.1869	0.1422	0.1989	40.35%
ERTB	15	7	0.0645	0.1614	0.0761	0.1745	16.67%
GOVI	2	2	0.0209	0.1108	0.0243	0.1278	3.51%
GRDO	10	10	0.0771	0.2019	0.0655	0.1705	14.04%
KAPEL	19	10	0.1128	0.197	0.1352	0.2055	32.89%
KLBE	2	2	0.0262	0.1233	0.0304	0.1423	4.39%
MELLE	14	8	0.1134	0.2158	0.1242	0.2135	26.75%
RIJK	3	3	0.0728	0.1996	0.0782	0.2094	12.28%
ROSK	24	14	0.1043	0.2107	0.0981	0.1920	24.56%
SLAA	14	6	0.0551	0.1451	0.0694	0.1783	13.60%
TENHN	2	2	0.0497	0.1665	0.0578	0.1920	8.33%
TENHZ	8	4	0.0393	0.1207	0.0541	0.1709	9.21%
VORS	5	5	0.052	0.1709	0.0509	0.1701	8.33%
WELL	8	7	0.0838	0.1993	0.0831	0.1823	17.98%
WIK1k	9	6	0.0439	0.1355	0.0588	0.1716	10.96%
WIK1	7	6	0.174	0.2246	0.2292	0.2719	42.98%
WIK11	7	6	0.0414	0.1313	0.0546	0.1668	10.09%
WIK12	8	8	0.2738	0.2551	0.3061	0.2525	63.60%
WIK13	1	1	-	-	-	-	-
WIK3	10	9	0.1065	0.1881	0.1410	0.2305	29.39%
WIK7	8	8	0.3483	0.2609	0.3890	0.2635	72.37%
ZWWA	5	3	0.0296	0.1231	0.0391	0.1531	6.14%
BOSV	29	13	0.0760	0.1334	0.1071	0.1781	29.74%
LANG1	27	27	0.1908	0.2066	0.1908	0.2066	62.83%
LANG2	2	2	0.0593	0.1942	0.0593	0.1942	8.55%
GOPL	1	1	-	-	-	-	-
RIJKP	25	19	0.1740	0.1785	0.1985	0.1981	59.11%
DAUT	18	3	0.0201	0.0807	0.0071	0.0670	1.12%
DOOD	30	30	0.2198	0.1638	0.2198	0.1638	80.67%
mean			**0.0996**		**0.1128**		25.97%
SD			**0.0802**		**0.0892**		22.75%

3.3 Genetic structure

The values of Φ_{PT} were 0.434, 0.296, 0.455 (p(rand ≥ data) = 0.001) for the dataset of 2009, 2010 and the combined dataset, respectively (clones excluded: 0.356, 0.226 and 0.365, respectively).

Figure 2 shows the PCoA based on the individual genetic distances of samples from 2009 (Figure 2 above) and 2010 (Figure 2 below), possible clones excluded. Samples of WIK7 and WIK12 show more variation in their scores along the principal axes than samples of other locations (Figure 2 above), confirming the aforementioned results. These locations are also clearly differentiated from other ones (Figures A 1 to 3 in Appendix 2). The samples group according to their origin, but some overlap is present. BEVE is well separated, but the samples from KAPEL and ERTB, and those of WIK (WIK7 and WIK12 not included), MELLE and TENHZ form mixed pools, even when additional PCoA axes are considered (results not shown). RIJKP and DAUT also form separate clusters (Figure 2 below), whereas BOSV and DOOD are more closely related. The PCoA of Nei's unbiased genetic distances between locations for the combined data excluding possible clones, shows that GOVI and WELL diverge most strongly from all other populations (Figure 3). According to the first and third PCoA axes (Figure A 3 in Appendix 2), WIK7 and WIK12 together are also set apart. Furthermore, Figure 3 (also Figure A 3, Appendix 2) shows that certain (sub)populations can be grouped according to their location: TENHN-TENHZ, KAPEL-ERTB, RIJKP-RIJK, WIK7-WIK12, all other WIK locations, LANG1-LANG2.

The genetic variation among locations of the combined dataset was 45% with potential clones and 36% without them (p(rand ≥ data) = 0.001). K-means clustering analysis of individuals suggested three clusters for the 2009 dataset and two for both the 2010 and combined dataset based on the pseudo-F statistic. However, the assignment of (sub)populations to the clusters differs between datasets. Based on BIC values, 16, 11 and 24 clusters were identified for each dataset, respectively. Except for the reference samples of 2009 added to the 2010 dataset, results were generally consistent. Moreover, analysis on the (sub)population level produced the same overall division in groups for both pseudo-F and BIC statistics. In the combined dataset, 14 clusters were suggested (Figure 4). The following locations belong to the same cluster: ERTB – KAPEL, WIK7 – WIK12, WIK1 – WIK1k – WIK11 – WIK13 – WIK 3, RIJK – RIJKP, LANG1 – LANG2 – GOVI - GRDO, MELLE – ZWWA – GOPL – TENHN – TENHZ, KLBE - SLAA and VORS – DOOD. Based on the results of the clustering of patches (K = 25), LANG1 – LANG2 are separated from GOVI – GRDO. This was also the case for WIK7 and WIK12, whereas two patches of DOOD and the single patch of VORS were still grouped together. However, the same could be said about DOOD and BOSV with two patches each in the same cluster. The two clusters containing geographically distant locations MELLE, ZWWA, GOPL and TENH – TENHZ on the one hand, and KLBE and SLAA on the other, still remained. Unexpectedly, a sampled patch of RIJKP containing free-floating individuals clustered separately when clustering was performed on the individual level or the patch level. A mixture of clusters occurred in locations DOOD, LANG1 and the WIK locations.

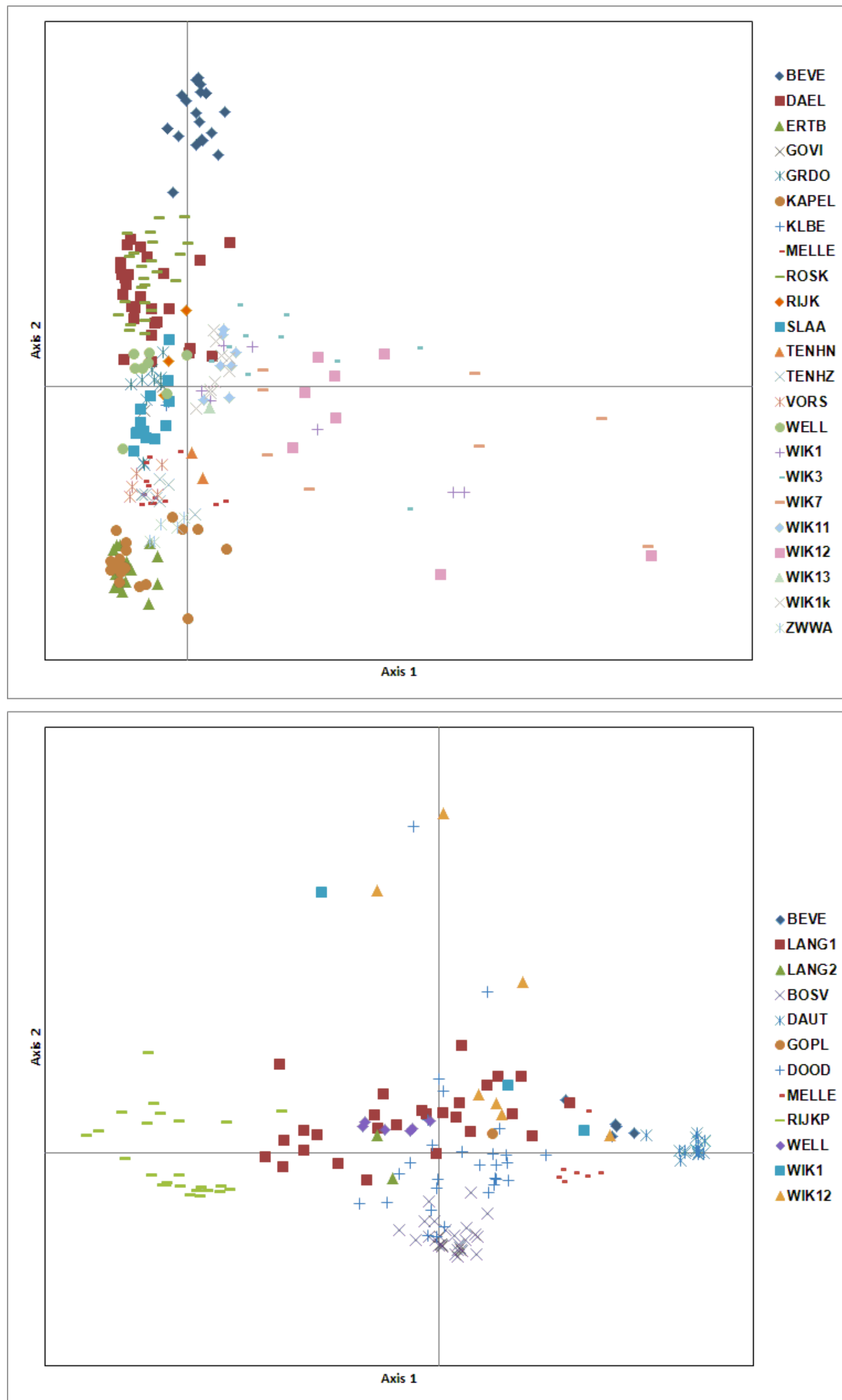

Figure 2 Principal coordinates analysis of Nei's genetic distances of *Luronium natans* samples from 2009 (above), based on 229 polymorphic AFLP markers with the first two axes respectively explaining 24.04 % and 21.24 % of the variation, and from 2010 (below), with 278 markers with the first two axes respectively explaining 30.62 % and 22.97 % of the variation.

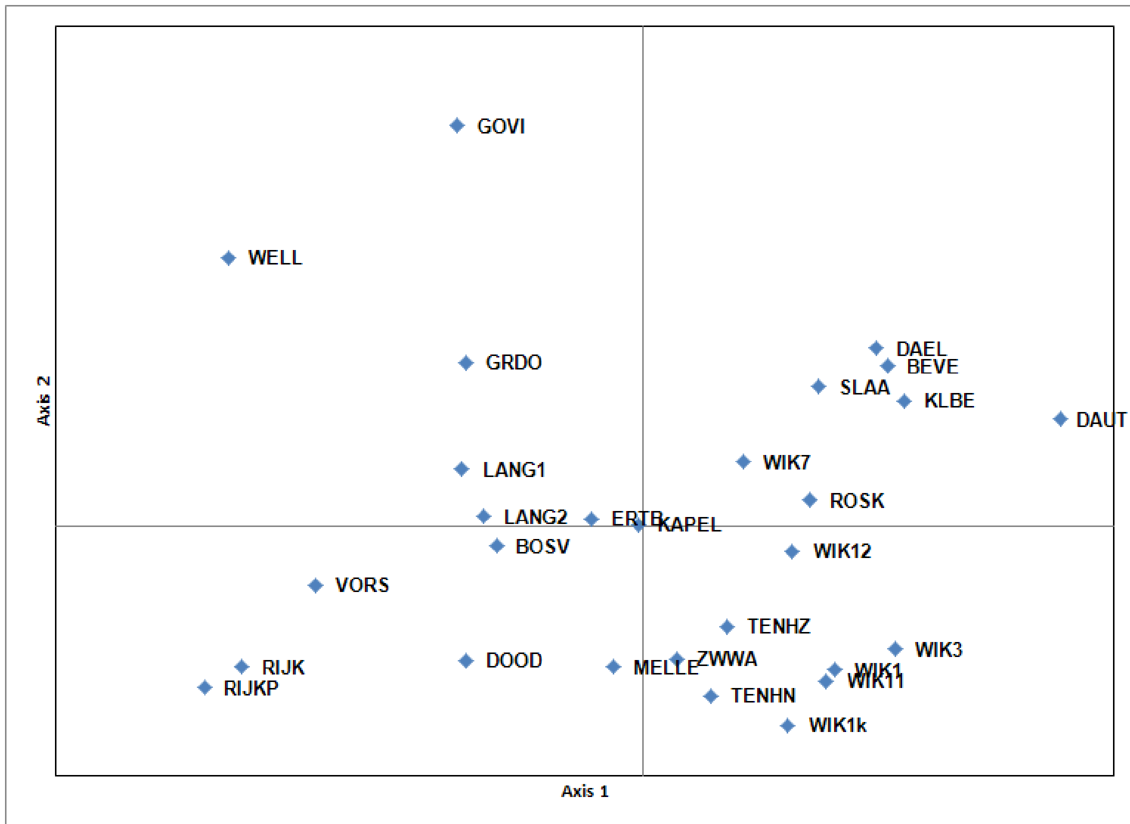

Figure 3 PCoA of Nei's unbiased genetic distances of all *Luronium natans* samples (unique ramets per subpopulation) based on 81 polymorphic AFLP markers with the first two axes explaining 27.61 % and 23.67 % of the variation, respectively.

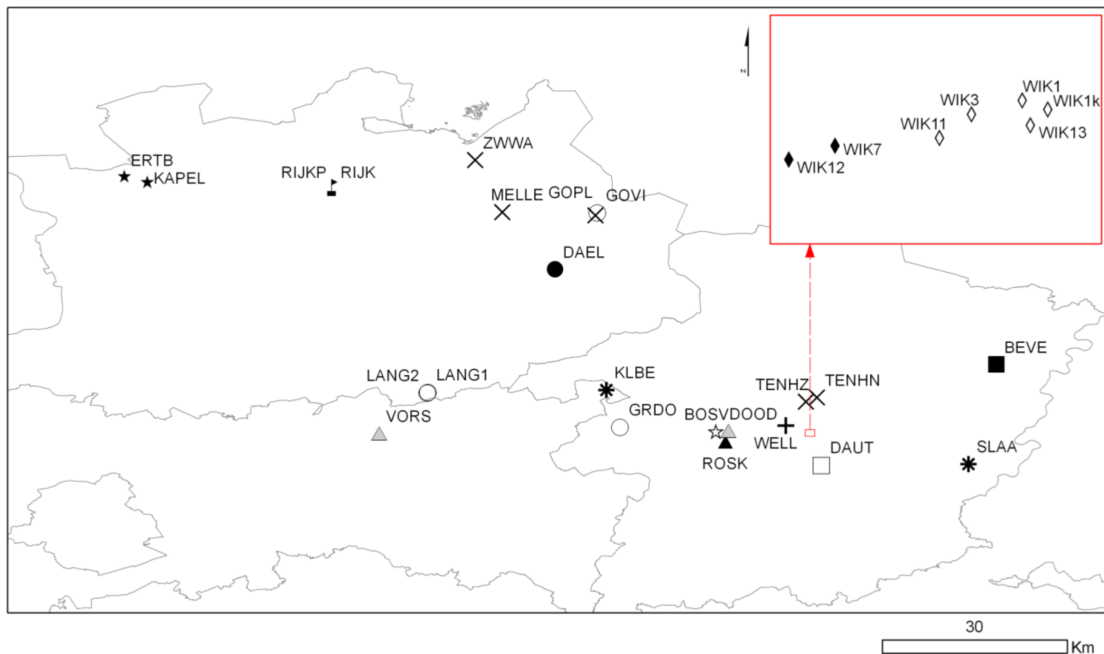

Figure 4 Results of K-means clustering of locations. Each symbol depicts a different group.

The results of the sPCA with maximum distance (d_{max}) equalling 0 or 10 km were very similar. We therefore only show the results for d_{max} = 10 and 45 km. In the first case, we

found a global structure (positive eigenvalues; p = 0.001) chiefly explained by the first three axes. There also appeared to be a local pattern (negative eigenvalues; p = 0.019) with only one important component. For d_{max} = 45 km, a global as well as a local pattern was detected (p = 0.009 and p = 0.036, respectively). The first principal component explained most of the global pattern, whilst the structure in local variability was largely fitted by one component. Based on the first prinicipal component for d_{max} = 10, a clinal structure emerges among the southern locations, whereas the northern locations are more or less divided into different groups (results not shown). Combining the first three axes gives a more clustered pattern that resembles the structure uncovered by K-means clustering, except that LANG1-LANG2 appears genetically similar to VORS, all WIK locations cluster together, and SLAA seems less similar to KLBE (Figure 5). In the local pattern, TENHN and DAUT are distinctly differentiated (Figure 6). With d_{max} = 45 km, the global structure represented a cline from east to west (Figure 7). The local pattern based on the last axis of the sPCA, differentiates population ERTB from KAPEL and clusters the other locations within a basin, except for the locations of the Nete basin (Figure 8).

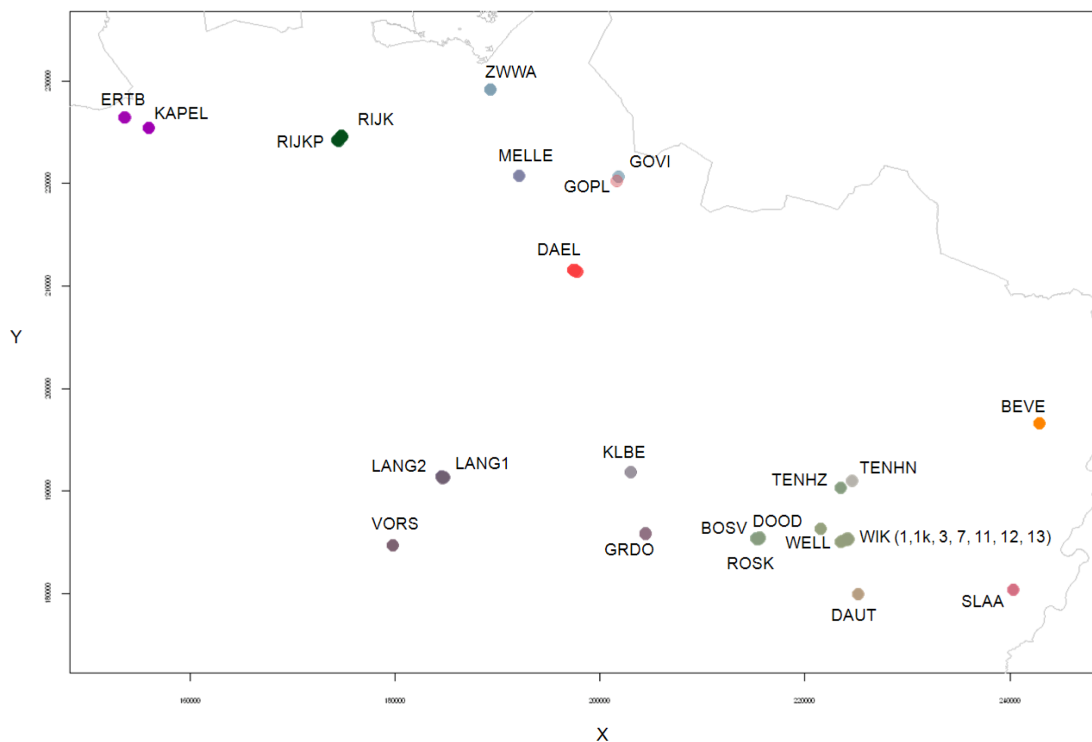

Figure 5 Spatial principal component analysis (sPCA) colourplot using lagged scores of the first three axes based on a neighbourhood by distance network with a minimum distance of zero and a maximum distance of 10 km.

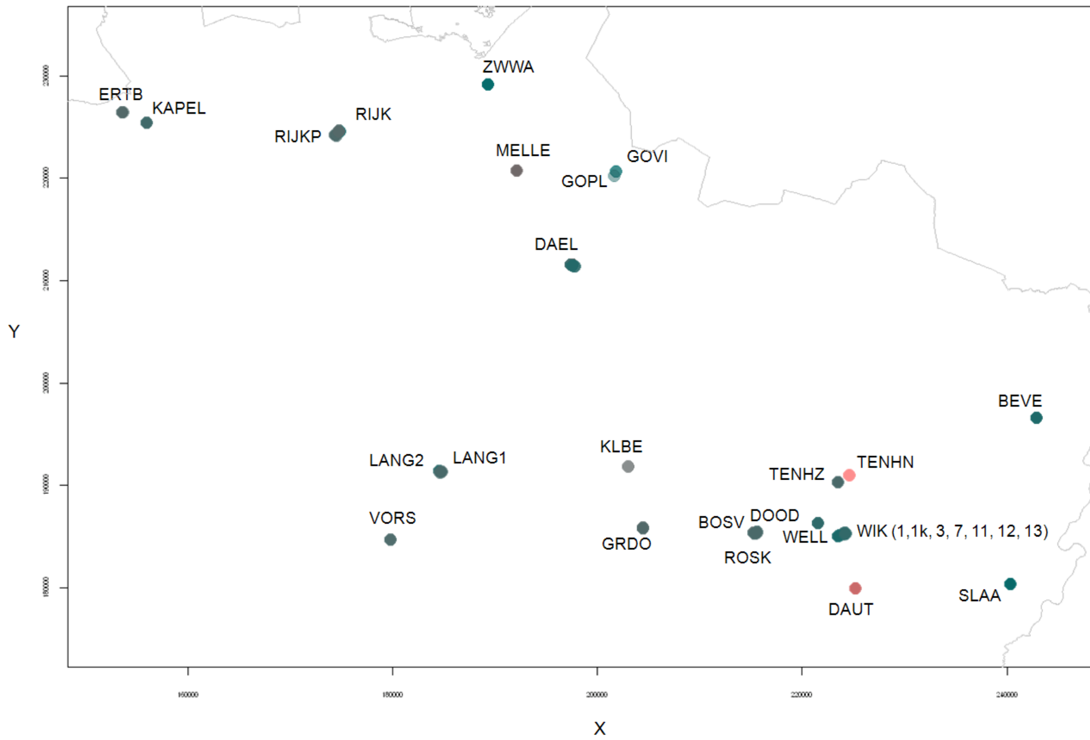

Figure 6 Spatial principal component analysis (sPCA) colourplot using lagged scores of the last axis based on a neighbourhood by distance network with a minimum distance of zero and a maximum distance of 10 km.

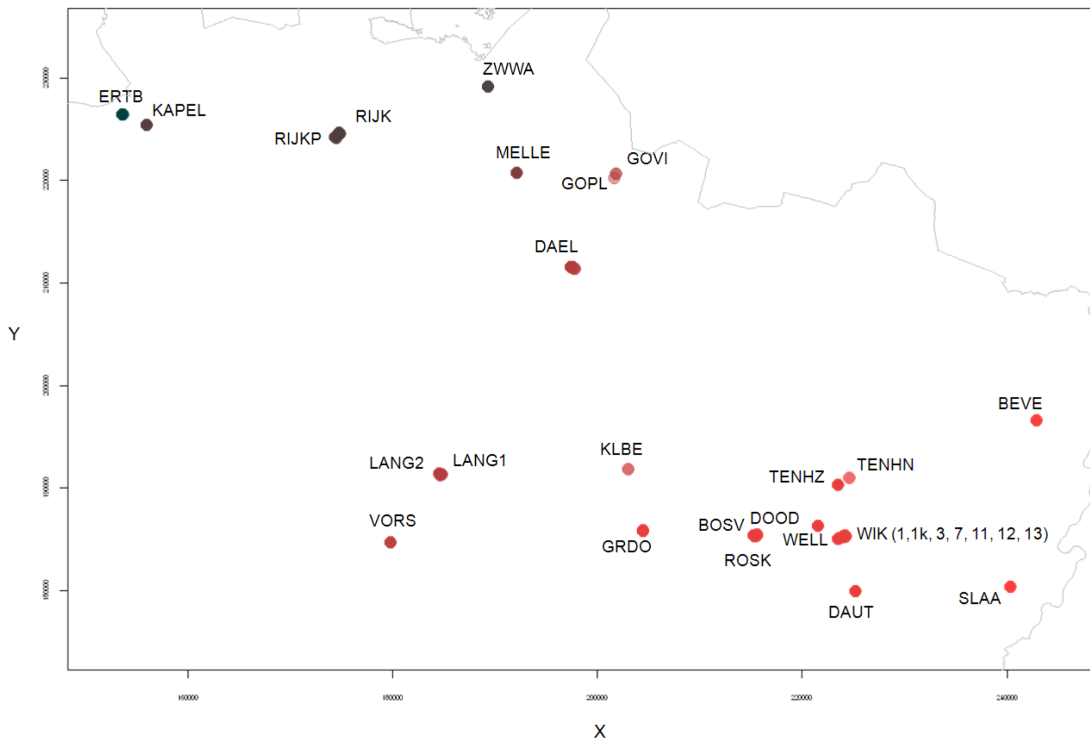

Figure 7 Spatial principal component analysis (sPCA) colourplot using lagged scores of the first axis based on a neighbourhood by distance network with a minimum distance of zero and a maximum distance of 45 km.

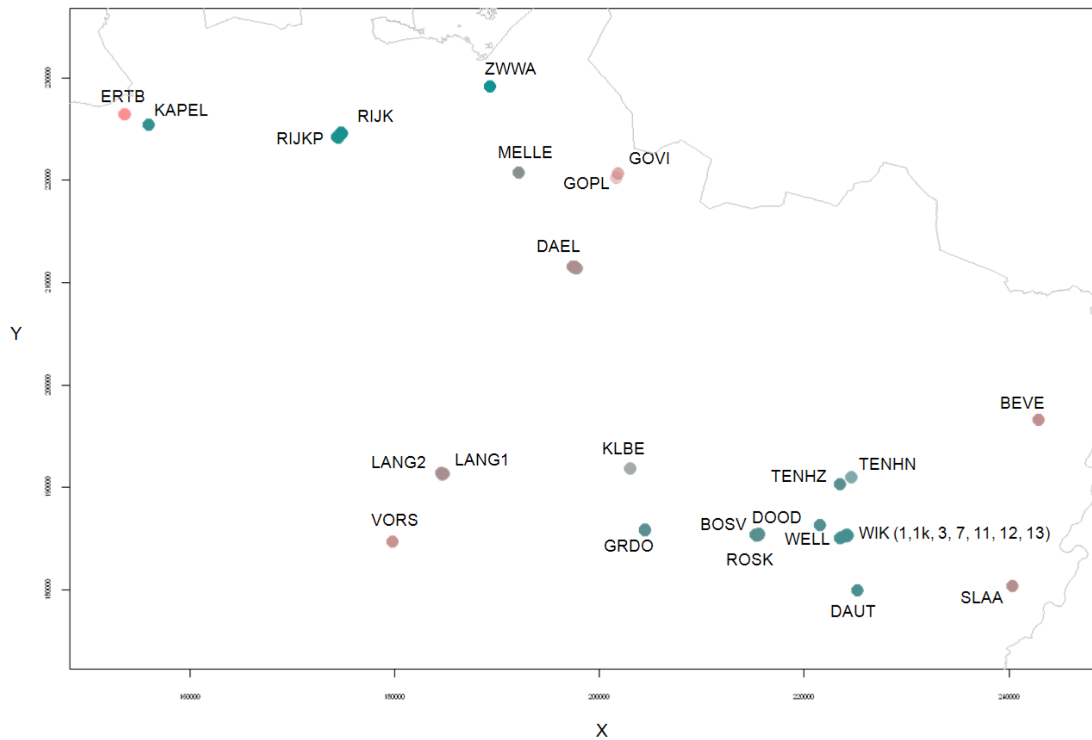

Figure 8 Spatial principal component analysis (sPCA) colourplot using lagged scores of the last axis based on a neighbourhood by distance network with a minimum distance of zero and a maximum distance of 45 km.

Mantel tests were significant for the data from 2009 (r = 0.386, p = 0.002) and 2010 (r = 0.470, p = 0.014). The correlation remained significant but weak for the combined dataset (r = 0.291, p = 0.001). The relationship was stronger at the Demer basin level (16 locations, r = 0.502, p = 0.001), but weaker in the Nete basin (8 locations, r = 0.367, p = 0.016). In addition to the low number of locations in the Nete basin, the slope of the linear relationship seems to be caused by two outliers (results not shown), making the Mantel test results for the Nete basin unreliable.

4 Discussion

4.1 Clonality and genetic diversity

We found a high degree of clonal reproduction in the investigated locations, as in Wales (Kay et al., 1999). Mostly, a dominant MLL was shared among patches within a water body. This is in line with expectations, as *Luronium natans* often reproduces vegetatively by runners (Barrat-Segretain et al., 1998; Willby & Eaton, 1993). Although clonal diversity was moderate to high, genetic diversity was generally low (e.g. mean *PPL* = 25%). Genotypic richness may have been overestimated, since it depends on the threshold used. Somatic mutations, technical and scoring errors may have increased the number of differences between actual ramets. Also, AFLP markers need to be adequately polymorphic to distinguish between highly related individuals and clones (Douhovnikoff & Dodd, 2003). We tested 32 primer combinations to gain sufficient resolution. Although the final six primer combinations proved useful, the majority of the markers (66% in 2009 and 61% in 2010) had allelic frequencies outside the range of 5 to 95%. Furthermore, our sampling design was inadequate to investigate the spatial structure of clones within populations, due to the small and linear nature of the transect (Arnaud-Haond et al., 2007) along which the majority of the samples were taken.

Low values for genetic diversity were found both in Denmark (Nielsen et al., 2006) and in Wales (Kay et al., 1999). In our case, small sample size may have prevented to capture the genetic variation of entire populations, in particular as sample size depended on census population size. Still, quite small samples revealed some of the higher values of the Shannon information index (*I*). For instance WIK1, with more than 1,000 plants, exhibited a high genetic diversity (*I* without clones = 0.23), on the basis of only seven samples. This supports our confidence in the observed pattern of genetic diversity among locations and its positive correlation with population size.

Since *L. natans* populations are polyclonal and produce abundant seeds with a high germination rate (Nielsen et al., 2006), a high level of genetic diversity would be expected, unless the populations were founded by one or a few plants. The seed bank can persist for more than 80 years (Janssen & Schaminée, 2004; Lucassen et al., 2007). Overlapping generations and long generation times due to strong seed bank dynamics can decrease the rate of genetic drift, thereby leading to a much longer retention of genetic diversity than expected in species without a seed bank or overlapping generations. Most of the locations had flowering plants (20 out of 28, excl. WIK7 and WIK12), but in 80% (16 out of 20) these represented only 0.5 to 5% of the total number of plants. As Lansdown & Wade (2003) mention, pollination can be limited when only a few plants are flowering at the same time. Pollen limitation in combination with clonal reproduction, could stimulate geitonogamous selfing in *L. natans*, mainly between ramets belonging to the same MLL. *L. natans* is presumed to be cleistogamous (Kay et al., 1999; Sculthorpe, 1967). However, submerged flowers were rare at the investigated sites, except where water-level changes had occurred. To gain insight in the contribution of the seed bank to the genetic diversity of populations, a sample of the seed bank should be genotyped and compared to the standing genetic diversity. Additionally, the genetic variation and makeup of the populations should be monitored periodically to assess if it changes considerably over time. The census population size can change dramatically between years, e.g. BOSV contained 1 to 10 plants in 2008, zero in 2009 and 100-1000 in 2010, and the number of plants in RIJK went from 100-150 in 2008 to ca. 20 plants in 2009. Clonal reproduction suffices for a sudden increase in the number of plants. So, it appears several populations were founded by one or a few migrants from neighbouring populations and were able to persist mainly through asexual reproduction. For example, van der Merwe et al. (2010) describe the persistence through time of an isolated founder population of a heterostylous shrub, *Erythroxylum pusillum*, through clonal spread and somatic mutations.

Certain MLLs were present at different locations. This was mainly on fairly close, connected sites, such as ERTB and KAPEL, both in the same canal and only 2.6 km apart, and cascading ponds in the nature reserve "Het Wik" (WIK1, WIK1k, WIK3, WIK11 and WIK13). Possibly, the MLL originated from WIK1-WIK1k, holding the largest and most diverse subpopulation, and travelled downstream for less than 100 m, illustrating that stolons disperse among interconnected water bodies (Greulich et al., 2000a). Unexpectedly, an MLL was also shared among ZWWA, MELLE and TENHZ, separated by about 10 km and 43.7 km. Although *L. natans* fragments can float for a long period enabling dispersal over large distances (Kay et al., 1999; Willby & Eaton, 1993), it can be excluded that they were transported solely by water in this case. Most likely dispersal occurred over land, e.g. by waterfowl, humans or both. Seed dispersal by birds was suggested to explain establishment of new populations in a lake 100 km from the nearest population site in Sweden (Fritz, 1989) and in a drainage canal 50 km away in England (Lansdown & Wade, 2003). Although stolons could also remain attached to the legs or feathers of waterfowl (Kay et al., 1999), their susceptibility to desiccation would limit transportation to shorter distances (Nielsen et al., 2006), while probably still accommodating for the reported distances.

Another possibility is the MLL found in ZWWA, MELLE and TENHZ originated from an intermediately positioned population that disappeared since. Clones can reach a high age. Those of the marine angiosperm, *Posidonia oceanica*, may even be more than 1,000 years old according to Hemminga & Duarte (2000).

4.2 Genetic structure

Populations of *Luronium natans* were highly differentiated. Genetic variation was also higher among than within populations for Welsh (Kay et al., 1999) and Danish populations (Nielsen et al., 2006). This is probably the result of genetic drift due to increased isolation and clonal reproduction with increasing biparental inbreeding. Naturally, limited dispersal contributes to the differentiation. The genetic structure found with K-means clustering and PCoA largely corresponds to the different populations, where KAPEL and ERTB, the WIK locations except WIK7 and WIK12, RIJK and RIJKP, and finally LANG1 and LANG2 cluster together as subpopulations in linked water bodies. Other clusters are KLBE-SLAA, GOVI-GRDO and ZWWA-MELLE-GOPL-TENHN-TENHZ. Except for TENHN-TENHZ, these do not correspond to hydrologically connected sites and comprise widely spread locations. Notably, the same MLL was found on all sites of the ZWWA-MELLE-GOPL-TENHN-TENHZ combination, except in TENHN and GOPL. According to the sPCA results with a d_{max} of 10 km or equal to d_{min}, a global pattern with a clinal relationship occurred among the sites in the Demer basin and a more patched pattern in the Nete and Scheldt basins. This suggests (former) connectivity, probably downstream in the Demer basin. An east to west clinal pattern was found when d_{max} was 45 km. Because dispersal over such distances appears uncommon, this pattern seems biologically less relevant, although such a longitudinal relationship might be of historical relevance. Genetic distance increased with geographic distance, suggesting isolation-by-distance, and even more so for the locations of the Demer basin, confirming the spatial pattern revealed by sPCA.

The ponds WIK7 and WIK12 act as sinks for stolons drifting from upstream locations within the Wik complex. Their high genetic diversity reflects the different origins of plants. However, if all the plants originated from other water bodies within this complex, WIK7 and WIK12 would cluster together with the other WIK locations. In fact, they are rather different, even from each other, in the PCoA and according to the K-means results. This points to different source populations for WIK7 and WIK12.

4.3 Genetic criteria for local conservation status

Although census population size and genetic diversity are related, clonal reproduction confounds this trend. In addition, genetic diversity seems low in general. Considering the strong population dynamics, periodical evaluation of the within-population genetic diversity is

necessary to gain insight in the importance of sexual reproduction and seed bank recruitment.

Structural connectivity between subpopulations does not seem to imply genetic connectivity in all cases. Locations WIK7 and WIK12 contained plant fragments that originated from the site itself or from outside the pond complex, whereas the other ponds were genetically well-connected. Still, as a general rule of thumb, structural connected subpopulations of *L. natans* can be perceived as a metapopulation and therefore as a single management/conservation unit.

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Appendix 1: list of multilocus lineages

Table A 1 List of samples of the same multilocus lineage (MLL) according to a Dice similarity of 0.98. Samples sharing the same character belong to the same MLL. The sample ID entails "location ID-patch number-sample number".

2009		**2010**	
Sample	**MLL**	**Sample**	**MLL**
BEVE-1-3	A	BOSV-1-1	K
BEVE-2-1	A	BOSV-1-2	K
BEVE-2-2	A	BOSV-1-4	K
BEVE-3-1	A	BOSV-1-5	K
BEVE-4-1	A	BOSV-1-6	K
BEVE-4-4	A	BOSV-1-7	K
BEVE-5-1	A	BOSV-1-8	K
BEVE-5-4	A	BOSV-1-9	K
BEVE-5-5	A	BOSV-2-1	K
BEVE-5-6	A	BOSV-3-1	K
BEVE-5-7	A	BOSV-3-2	K
DAEL-1-1	B	BOSV-3-3	K
DAEL-2-1	B	BOSV-4-1	K
DAEL-2-3	B	BOSV-4-2	K
DAEL-3-1	B	BOSV-5-2	K
DAEL-4-1	B	BOSV-7-2	K
DAEL-4-2	B	BOSV-8-3	K
DAEL-4-5	B	DAUT-1-1	L
DAEL-4-9	B	DAUT-1-2	L
DAEL-4-10	B	DAUT-1-4	L
DAEL-4-11	B	DAUT-1-5	L
DAEL-4-12	B	DAUT-1-6	L
DAEL-4-13	B	DAUT-2-1	L
DAEL-4-14	B	DAUT-2-2	L
DAEL-3-2	C	DAUT-2-3	L
DAEL-3-3	C	DAUT-4-1	L
ERTB-1-1	D	DAUT-5-1	L
ERTB-2-1	D	DAUT-6-1	L
ERTB-2-4	D	DAUT-7-1	L
ERTB-3-1	D	DAUT-8-1	L
ERTB-3-2	D	DAUT-8-2	L
ERTB-4-1	D	DAUT-9-1	L
ERTB-4-2	D	DAUT-9-2	L
ERTB-8-2	D	RIJKP-4-2	M
ERTB-8-4	D	RIJKP-5-1	M
KAPEL-1-1	D	RIJKP-7-1	M
KAPEL-1-3	D	RIJKP-7-2	M
KAPEL-1-4	D	RIJKP-15-1	M
KAPEL-1-5	D	RIJKP-5-2	N
KAPEL-1-8	D	RIJKP-6-1	N
KAPEL-1-9	D	RIJKP-10-1	N
KAPEL-1-10	D		
KAPEL-1-16	D		
KAPEL-1-21	D		
KAPEL-1-23	D		
MELLE-1-1	E		
MELLE-1-3	E		
MELLE-2-1	E		
MELLE-2-3	E		
MELLE-3-2	E		

2009		2010	
Sample	**MLL**	**Sample**	**MLL**
MELLE-4-1	E		
MELLE-4-2	E		
TENHZ-1-2	E		
TENHZ-2-3	E		
TENHZ-2-4	E		
TENHZ-2-5	E		
TENHZ-3-1	E		
ZWWA-0	E		
ZWWA-1-3	E		
ZWWA-1-4	E		
ROSK-2-3	F		
ROSK-4-1	F		
ROSK-5-3	F		
ROSK-5-8	F		
ROSK-5-10	F		
ROSK-5-14	F		
ROSK-5-15	F		
ROSK-5-17	F		
ROSK-5-18	F		
ROSK-5-23	F		
SLAA-1-1	G		
SLAA-1-2	G		
SLAA-1-4	G		
SLAA-1-6	G		
SLAA-2-1	G		
SLAA-2-2	G		
SLAA-3-1	G		
SLAA-3-3	G		
SLAA-3-6	G		
WELL-1-1	H		
WELL-1-10	H		
WIK1-6-1	I		
WIK1-7-1	I		
WIK3-1-1	I		
WIK3-6-1	I		
WIK11-5-2	I		
WIK11-5-3	I		
WIK13-1-1	I		
WIK1k-4-1	I		
WIK1k-5-1	I		
WIK1k-5-2	I		
WIK1k-5-4	J		
WIK1k-5-5	J		

Appendix 2: PCoA plots

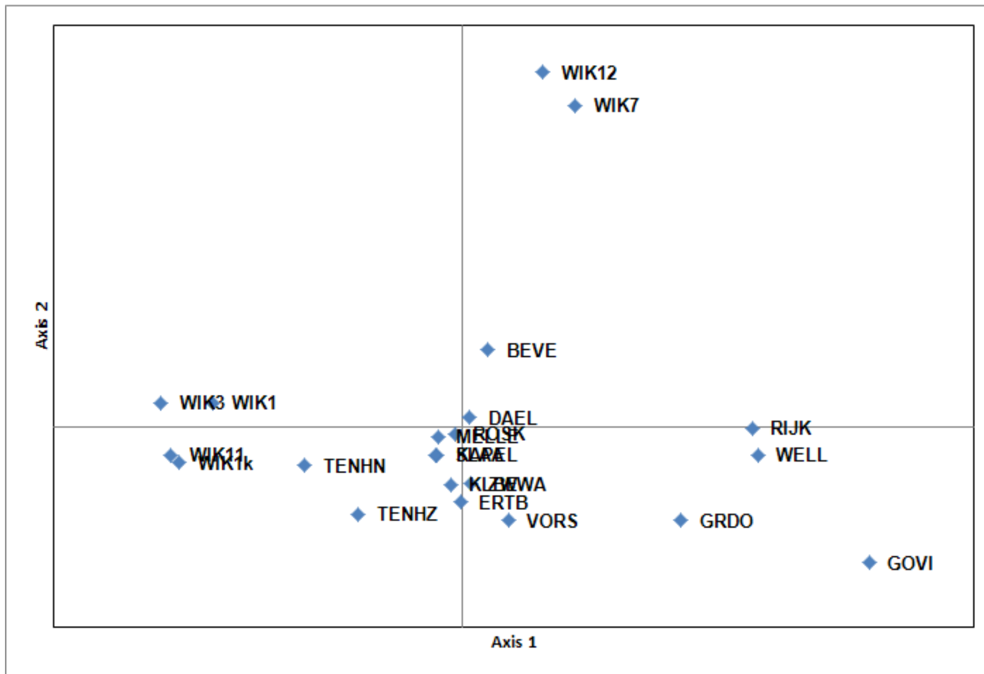

Figure A 1 PCoA of Nei's unbiased genetic distances of *Luronium natans* samples from 2009 (unique ramets per subpopulation) based on 228 polymorphic AFLP markers with the first two axes explaining 26.29 % and 23.96 % of the variation, respectively.

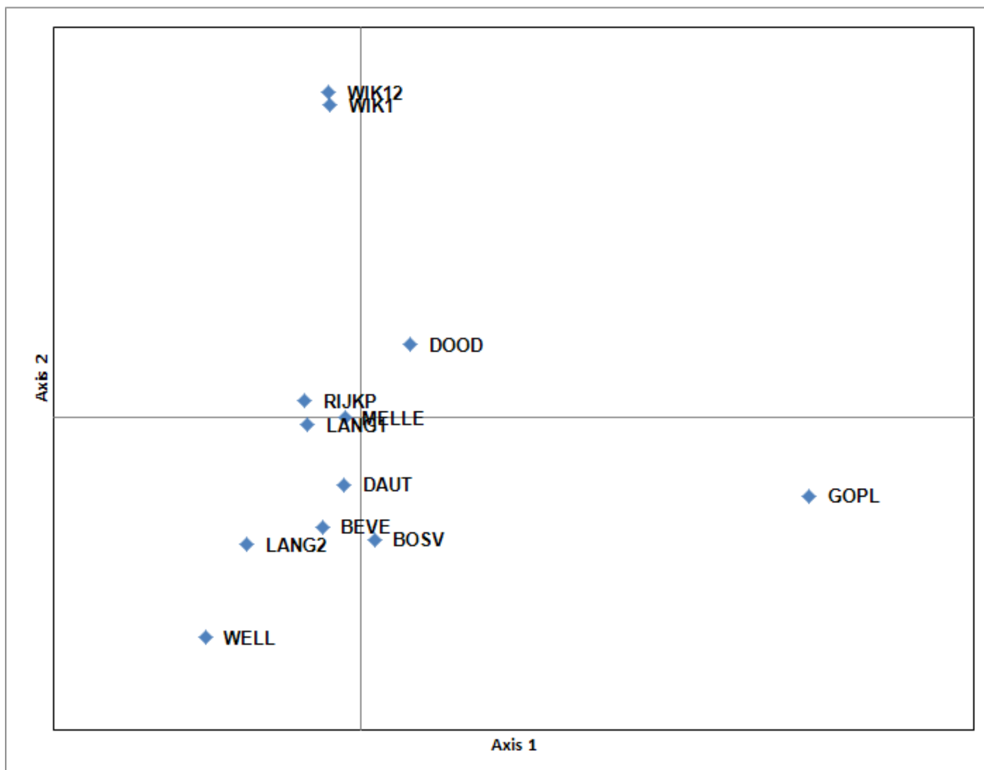

Figure A 2 PCoA of Nei's unbiased genetic distances of *Luronium natans* samples from 2010 (unique ramets per subpopulation) based on 278 polymorphic AFLP markers with the first two axes explaining 29.47 % and 22.49 % of the variation, respectively.

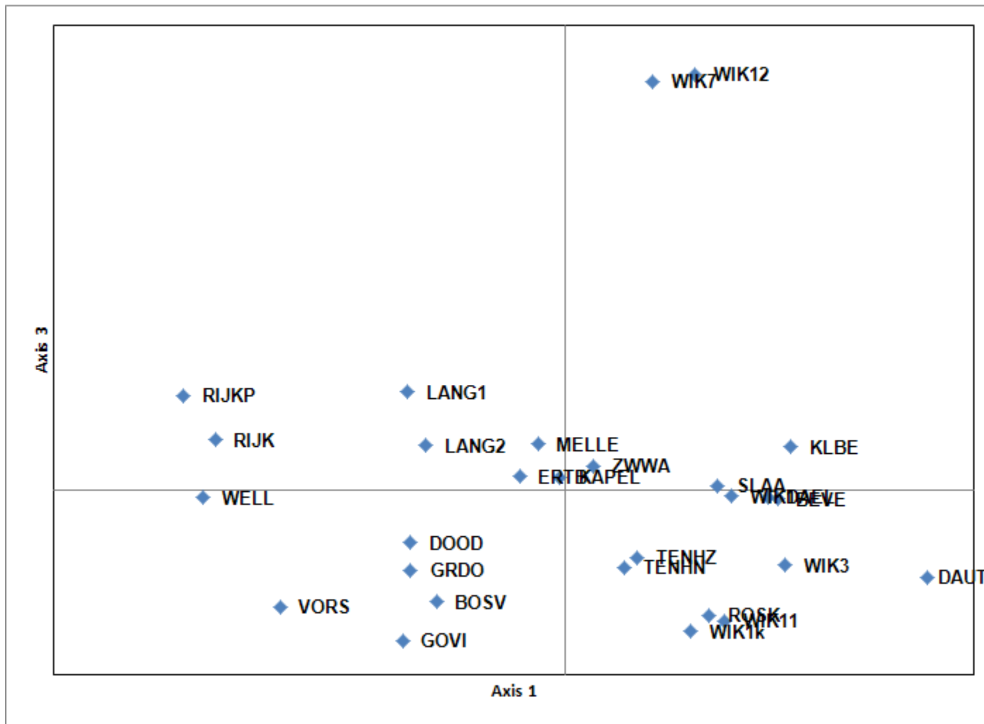

Figure A 3 PCoA of Nei's unbiased genetic distances of all *Luronium natans* samples (unique ramets per subpopulation) based on 81 polymorphic AFLP markers with the first and third axes explaining 27.61 % and 15.53 % of the variation, respectively.