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Short communication

The importance of vegetative and sexual dispersal of *Luronium natans*

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Abstract

Luronium natans (L.) Rafin. is a very rare macrophyte even though it has the ability to grow in a wide variety of habitat types. Previous studies leave two possibilities for this pattern: the species has a poor ability to disperse and establish or it is unable to grow and develop a sustainable population after colonization.

Experiments on establishment of shoots, seed bank dynamics, seed germination and genetic analysis (AFLP) were conducted to establish whether *L. natans* disperse to new habitats within hydrologically linked water systems by means of vegetative shoots or by seeds. Shoots had high ability to establish by roots (52% in autumn shoots), but only when subjected to water depths <4 cm. Seeds of *L. natans* has a high germinate rate (mean of 51–60%), and the density of seeds in the seed bank ranged from 635 to 3354 m^{-2} during a year. Analysis of the genetic diversity showed that samples could be differentiated to individuals with higher diversity between populations than within population. Low ability of shoots to establish if not subjected to low water depth, high germination rate of seeds, substantial seed bank and a high genetic diversity all indicate that most colonization events depend on sexual reproduction.

Genetic diversity in *L. natans* seems to depend on habitat type and management. Habitats subjected to high water velocities or management with weed cutting generally have a lower genetic diversity (5–6%) than habitats subjected to dredging (11%), indicating that the latter habitats favor sexual reproduction.

Keywords: *Luronium natans*; Genetic diversity; Seed bank; Seed germination; Vegetative reproduction; Sexual reproduction

1. Introduction

Aquatic plants have evolved different means of reproduction and dispersal in order to be able to spread within and between aquatic systems. Most aquatic plant species spread by hydrochory (dispersal by water) as opposed to terrestrial plant species which spread by anemochory (dispersal by wind) or zoochory (dispersal by animals) (Sculthorpe, 1967; Barrat-Segretain, 1996; Greulich et al., 2000a). When an aquatic plant reach a lake or a river it is preferable to remain in the habitat and this is secured with hydrochory. However, hydrochory represents a limitation in dispersal between water systems, and only dispersal within lakes and downstream rivers can be expected.

Moreover, the aquatic environment has favored the development of more or less specialized vegetative propagules (e.g. turions, bulbils) that can disperse by hydrochory within systems. Some of these are able to survive unfavorable conditions as winter or drought and establish under favorable conditions. The importance of vegetative dispersal can be substantial and a study with colonization of *Callitriche cophocarpa* Sendtner in a Danish stream showed that 90% of all new colonizations were due to drift of vegetative fragments (Sand-Jensen et al., 1999).

Luronium natans is a rare macrophyte endemic to Europe. Though rare it is found in very different habitat types ranging from oligotrophic lakes to eutrophic canals. It seems that the natural habitat is oligotrophic lakes (Hanspach and Krausch, 1987; Roelofs, 1996; Szankowski and Klosowski, 2001), but it is tolerant of disturbances and is, hence, also found in rivers with high disturbance regimes (Willby and Eaton, 1993; Greulich et al., 2000b).

L. natans has four different growth forms which predominates in different habitats: it is found as a small isoetid in the deeper parts of lakes, as a nymphaeid with floating leaves in shallow waters of lakes and rivers, as a rosette with long linear leaves in deep areas of slow flowing, larger rivers and as a little terrestrial plant (Kay et al., 1999). The floating leaf type usually

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occurs in shallow water, but is found submerged as well as on the water surface. Hence, it is more appropriate to use the term expanded leaves instead of floating leaves (Lansdown and Wade, 2003). Lateral propagation occurs by pseudostolons (runners formed by differentiated inflorescences). New shoots (small rosettes) are formed on the pseudostolons and develop into new plants. In some cases the pseudostolons do not attach to the sediment and the shoots are formed in the water column. The shoots break of easily and can act as vegetative propagules that disperse and colonize new habitats (Willby and Eaton, 1993).

There is a lack of information on the dispersal and colonization of *L. natans*. Willby and Eaton (1993) described it as an early colonizer, but an experiment by Barrat-Segretain and Amoros (1996) showed that it was one of the last species to colonize cleared patches in a French River. Recent research has reported high survival rate of *L. natans* shoots, which would help dispersal within water systems (Barrat-Segretain and Bornette, 2000), but studies have failed to show significant establishment by shoots (Barrat-Segretain et al., 1998, 1999; Barrat-Segretain and Bornette, 2000). The establishment of vegetative shoots is probably affected by factors, such as water depth, temperature, season and nutrition.

A large production of seeds should result in a high probability to reach unoccupied habitats and promote colonization of *L. natans* in new habitats (Thompson et al., 2002). However, the success of colonization by seeds is influenced by dispersal, seed bank dynamics, germination rate of the seeds and physical and chemical conditions needed for germination.

The objective of this study was to assess to which extent *L. natans* disperse and colonize by vegetative shoots or by sexual reproduction. We studied the establishment ability of shoots, the seed bank dynamics, the germination rate of seeds and the genetic distances within and between Danish populations.

2. Materials and methods

2.1. Study area and plant material

In Denmark *L. natans* is only found in a few canals and lakes in a limited area close to the west coast of Jutland. Five locations with populations of *L. natans* were used in the study. Lake Husby (UTM zone32/euref89: 6236157-451114) is an 87 ha shallow lake with a small (approximately 300 m^2) population of *L. natans* with only submerged leaves at about 0.9 m depth. Kimmelkær Canal (6227400-451000) is a 2 m wide drainage canal (maximum depth approximately 1 m) with very low or no water flow most of the year. The canal is managed regularly by sediment dredging. Southern Parallel Canal (6197901-46938; width ranging from 5 to 20 m) is a tributary to the River Skjern. Southern Parallel Canal has very low water flow most of the time, and is managed by weed cutting twice a year. In River Skjern, the largest river in Denmark, *L. natans* is found in the lower reaches of the river (6197836-470177). The river was restored in 2000 and two small populations of *L. natans* were found at the bank of the restored river in 2003. Gødelen (6187500-451000) is a drainage canal with relative low water flow and a width of 8 m.

2.2. Establishment of vegetative shoots

To assess the establishment ability of vegetative shoots of *L. natans* newly formed shoots were collected from Kimmelkær Canal and Lake Husby in the summer (June) and in the autumn (October), and subjected to two different water depths in the laboratory. The shoots were all formed on pseudostolons and, though having produced roots, had not yet attached to the sediment. In summer the shoots were grown at 20 °C and a 15-h light/9-h dark cycle. Eight shoots from either Kimmelkær Canal or Lake Husby were placed in each of six trays (4.5 cm × 26 cm × 29 cm) with water depths ranging from 2 to 4 cm due to evaporation, and eight shoots were placed in each of 12 aquariums (14 cm × 19 cm × 29.5 cm) with water depth ranging from 10 to 12 cm. In autumn only shoots from Kimmelkær Canal were used, and the shoots were grown at 12 °C and a 12-h light/12-h dark cycle for 13 weeks. Light intensity at the plant level was >300 μmol m^{-2} s^{-1}. Establishment of the shoots was checked once a week during the experiment and successful establishment was defined as rooting to a depth of 1 cm into the sediment.

2.3. Seed bank and germination

The seed bank in Kimmelkær Canal was monitored three times from June 2003 to April 2004 to determine annual dynamics. The first samples were taken 26/6 after spring germination and before flowering in 2003. Autumn samples were taken 31/10-2003 and spring samples were taken 26/4 just before germination in 2004. Three sample sites just downstream a population of *L. natans* in the canal was chosen and ten bottom samples were taken at each site at every collection date. The upper 10 cm of sediment was collected with a Kajak tube with a diameter of 5.3 cm, resulting in a total collection area of 220.6 cm^2 and a volume of 2206 cm^3. The samples were washed in a 40 μm mesh sieve to remove fine organic particles and mud and then hand sorted and counted.

All intact seeds were placed in petri dishes (one dish per sample site per sample date; n = 14–74) and incubated at 12 °C with a diurnal 15-h light/9-h dark cycle for germination. The number of germinated seeds was counted twice a week for 8 weeks. Germination was successful when the radicle was observed. The first case of germination was observed after only a couple of days, peaked within 15 days and then stopped.

2.4. Genetic diversity

Genetic diversity between individuals from different populations can be used to assess whether *L. natans* colonize new habitats by vegetative propagules or seeds. Plant samples were collected at the end of the growth season in October 2003 from five different locations. At each location one to three populations were sampled. Here, a population is defined as a

Table 1
Number of populations and of plant samples within populations, and distance between populations within locations and between samples within populations used for analysis of genetic diversity

Location	Number of populations	Number of samples per population	Distance between populations within locations (m)	Distance between samples within populations (m)	Genetic distance within location (%)
Lake Husby	1	4	–	5	$3.97^{d} \pm 0.55$
Kimmelkær Canal	3	4 + 4 + 4	50	1.5	$10.78^{a} \pm 1.18$
Southern Parallel Canal	3	4 + 4 + 3	50	2	$6.29^{b} \pm 1.08$
River Skjern	2	4 + 4	500	1.5	$4.51^{d} \pm 0.37$
Gødelen	3	4 + 4 + 4	50	4	$5.36^{c} \pm 0.81$

Mean (±S.D.) genetic distance between individuals within a location. Superscripts (a–d) indicate significant difference (Kruskal–Wallis, $p < 0.001$).

single coherent group of plants. Three populations were sampled where possible. Lake Husby and River Skjern had only one and two populations, respectively. Within each population 4 (in one case 3) samples were taken resulting in a total of 12 samples from Kimmelkær Canal and Gødelen, 11 samples from Southern Parallel Canal, 8 samples from River Skjern and 4 samples from Lake Husby. Due to different population sizes among locations, plant samples were taken at different distances within populations from different locations (Table 1).

The populations in Kimmelkær Canal, Southern Parallel Canal and Gødelen were collected with approximately 50 m between populations while the two populations in River Skjern were located at the same bank approximately 500 m apart. A total of 12 populations and 47 individual samples were analyzed (Table 1). Only young, light green leaves were used to avoid contamination from epiphytes. The leaves were gently cleaned in tap water and then frozen at −18 °C until used for DNA-extraction.

DNA extraction was done by the C-TAB method described in Stewart and Via (1993), with the following changes: leaf samples were homogenised with liquid N_2, 400 μl CTAB and 2 μl proteinase were added and incubated at 60 °C for 1 h. Then 400 μl chloroform-isoamyl alcohol (24:1, v/v) was added, mixed and centrifuged in 2 min (13,000 rpm). The aqueous phase was transferred to a fresh micro tube, precipitation was done with 1 volume isopropanol, placed in a freezer at −20 °C for half an hour and centrifuged in half an hour (13,000 rpm). The pellet was air dried for 30 min and resuspended in 40 μl TE-buffer.

In this study, Amplified Fragment Length Polymorphisms (AFLP) is used to determine the genetic distance within locations and within and between populations of *L. natans* (Vos et al., 1995). The strength of this method lies in the fact that no former knowledge is needed about the genome of the species in interest. Furthermore, it is a reproducible method and it yields a high quantity of characters (on average about 100 bands per gel), which makes it particularly good for determining genetic variation within species (Karp et al., 1996).

AFLP uses restriction enzymes to cut the DNA into smaller fragments from which specific fragments are chosen using primers with three specific base pairs. The restriction enzymes *Eco*RI and *Mse*I were used to cut the DNA. To find primer combinations to achieve the best results 36 combinations of selective primers were tested. The three combinations, which produced the best curves and peaks, were selected (*Eco*RI-CGT + *Mse*I-ACG; *Eco*RI-CAG + *Mse*I-CCG and *Eco*RI-CAG + *Mse*I-CTA).

The AFLP occurs in three steps: annealing and ligation of adaptors, preamplification and selective amplification. All three steps were run on a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA). After this the products were run by electrophoreses on an acryl amid gel using an ALFexpressII machine (Amersham Pharmacia, Biotech) and then analysed using the program ALFwin Fragment Analyser 1.0.

The results were given as a curve with peaks indicating fragments of different length on an ALFexpress machine. The peaks were scored for each sample as present or absent (1 or 0) and used as characters (a total of 210 characters). From these the genetic diversity could be calculated. Genetic distances within and between populations, and within and between locations were calculated as the mean difference in characters between individuals from one population or location to all individuals in another population or location. Afterwards the results were transformed to percentage.

To test the reproducibility of the results from the ALFexpress six samples were duplicated and run through the whole process.

2.5. Statistics

Differences between means were analyzed by one-way ANOVA. In cases of variance inhomogeneity the non-parametric Kruskal–Wallis test was used. Both tests were run using StatGraphics Plus 4.1 (Manugistics, Inc., MD, USA).

3. Results

3.1. Establishment of vegetative shoots

Shoots of *L. natans* collected in summer and in autumn only established by roots when subjected to water depths <4 cm. There were statistical significant difference between establishment ability of shoots collected in summer and shoots collected in autumn (Kruskal–Wallis, $p < 0.05$). Mean (±S.D.) establishment of shoots collected in summer were 9.4% (±9.4) while mean (±S.D.) establishment ability of shoots collected in autumn were 52% (±24). No shoots established when subjected to water depth >10 cm.

Table 2
Mean (±S.D.) density of seeds in Kimmelkær Canal and mean (±S.D.) germination rate of the seeds in the laboratory

	Density (seeds m^{-2})	Germination rate (%)
Summer 2003	907 ± 297^{a}	59 ± 21^{a}
Autumn 2003	2492 ± 910^{b}	60 ± 12^{a}
Spring 2004	2584 ± 635^{b}	51 ± 11^{a}

Superscripts (a and b) indicate significant difference between seasons (ANOVA, $p < 0.05$).

3.2. Seed bank dynamic and seed germination

The seed density in Kimmelkær Canal varied between 635 and 3354 m^{-2} with mean (±S.D.) abundance ranging from 2584 m^{-2} (±635) in spring before germination to 907 m^{-2} (±297) in summer after germination (Table 2). The germination rate of seeds found in the sediment samples varied between 36 and 75% during the experiment. Mean germination rate did not differ significantly between summer, autumn and spring seeds (Table 2).

3.3. Genetic diversity

All plant samples could be differentiated in term of genetic diversity indicating that they originate from different individuals. Some of the samples were, however, very similar and two samples differed by only two characters. The results indicate that sexual reproduction is of high significance in all of the sample sites.

When measured as the overall variation within a location, Lake Husby and River Skjern had the lowest genetic variation, while the highest genetic variation was found in Kimmelkær Canal (Table 1). When genetic variation was calculated within populations the lowest genetic variation was found within population 1 in River Skjern and the highest in population 3 in Kimmelkær Canal (Table 3).

4. Discussion

4.1. Dispersal and establishment by vegetative shoots

Vegetative colonization of new habitats can occur by two means. New habitats and cleared patches can be colonized randomly by drift of propagules (propagule propagation) or colonization can occur from the surrounding vegetation by runners or pseudostolons (peripheric propagation). The latter is known as the border effect and has proven to be an important colonization method for *L. natans* (Barrat-Segretain and Amoros, 1996). The pseudostolons and inflorescences of *L. natans*, on which new shoots are formed, are very fragile and break off easily, and this releases a lot of floating shoots and could result in propagule propagation.

In this study, we found that shoots of *L. natans* collected in summer and in autumn only were able to attach to the sediment when subjected to water depths <4 cm. In previous studies only limited colonization ability by shoots of *L. natans* has been found (Barrat-Segretain et al., 1998; Barrat-Segretain and Bornette, 2000), probably because deeper water (7 cm) was used in the studies. Shoots of *L. natans* rarely sink and will not gain contact with the sediment at high water levels and, hence, no establishment can occur.

The fact that *L. natans* only colonize if subjected to very shallow water levels indicates that if a shoot reaches a site where it gains contact with the sediment it will most likely be able to grow at the site. However, this is complicated by the fact that most riverbanks and lakeshores would be expected to have existing plant communities and that these will compete with the shoot. Wilson and Keddy (1991) found that neighboring plants affect establishment and survival of fragments of macrophytes, while established plants are not affected by competition from other individuals.

More of the shoots collected in autumn established than shoots collected in summer. This seems to be an effect of lowered shoot and flower production in autumn conditions due

Table 3
Mean genetic distance (%) within populations and between populations measured as genetic distance from each sample from one population to samples in another

	H ($n = 4$)	K1 ($n = 4$)	K2 ($n = 4$)	K3 ($n = 4$)	SP1 ($n = 4$)	SP2 ($n = 4$)	SP3 ($n = 3$)	S1 ($n = 4$)	S2 ($n = 4$)	G1 ($n = 4$)	G2 ($n = 4$)	G3 ($n = 4$)
H	**4.0^{a}**	17.2^{b}	9.1^{a}	14.9^{b}	8.8^{b}	9.6^{b}	9.9^{b}	9.9^{b}	7.5^{b}	8.7^{b}	7.9^{b}	9.2^{b}
K1	17.2^{b}	**8.9^{a}**	13.2^{b}	10.8^{a}	14.2^{b}	14.5^{b}	13.8^{b}	14.8^{b}	13.7^{b}	14.6^{b}	14.8^{b}	15.7^{b}
K2	9.1^{b}	13.2^{b}	7.7^{a}	10.9^{a}	8.3^{b}	8.6^{a}	8.0^{a}	10.4^{b}	7.6^{b}	7.7^{b}	7.1^{b}	9.8^{b}
K3	14.9^{b}	10.8^{b}	10.9^{b}	**10.7^{a}**	12.8^{b}	13.4^{b}	12.0^{b}	14.5^{b}	12.6^{b}	12.9^{b}	12.9^{b}	14.5^{b}
SP1	8.8^{b}	14.2^{b}	8.3^{a}	12.8^{b}	**6.1^{a}**	6.6^{a}	6.6^{a}	11.2^{b}	7.4^{b}	7.0^{b}	6.7^{b}	8.5^{b}
SP2	9.6^{b}	14.5^{b}	8.6^{a}	13.4^{b}	6.6^{a}	**5.8^{a}**	6.1^{a}	11.1^{b}	7.1^{b}	7.4^{b}	7.9^{b}	9.0^{b}
SP3	9.9^{b}	13.8^{b}	8.0^{a}	12.0^{b}	6.6^{a}	6.1^{a}	**5.4^{a}**	10.1^{b}	6.7^{b}	7.0^{a}	6.7^{b}	9.6^{b}
S1	9.9^{b}	14.8^{b}	10.4^{b}	14.5^{b}	11.2^{b}	11.1^{b}	10.1^{b}	**2.7^{a}**	5.8^{b}	11.3^{b}	10.5^{b}	12.1^{b}
S2	7.5^{b}	13.7^{b}	7.6^{a}	12.6^{b}	7.4^{a}	7.1^{a}	6.7^{a}	5.8^{b}	**2.9^{a}**	7.5^{b}	6.9^{b}	8.5^{b}
G1	8.7^{b}	14.6^{b}	7.7^{a}	12.9^{b}	7.0^{a}	7.4^{b}	7.0^{a}	11.3^{b}	7.5^{b}	**4.2^{a}**	4.4^{a}	6.6^{b}
G2	7.9^{b}	14.8^{b}	**7.1^{a}**	12.9^{b}	6.7^{a}	7.9^{b}	6.7^{a}	10.5^{b}	6.9^{b}	4.4^{a}	**2.8^{a}**	7.3^{b}
G3	9.2^{b}	15.7^{b}	9.8^{b}	14.5^{b}	8.5^{a}	9.0^{b}	9.6^{b}	12.1^{b}	8.5^{b}	6.6^{b}	7.3^{b}	**3.0^{a}**

H = Lake Husby; K = Kimmelkær Canal; SP = Southern Parallel Canal; S = River Skjern; G = Gødelen. Superscipts (a and b) indicate significant difference within columns (Kruskal–Wallis, $p < 0.05$). Bold marks the genetically most similar populations within a column.

to increased allocation to root formation. Accordingly Barrat-Segretain and Bornette (2000) found that spring fragments develop new shoots, while root development only occurs in autumn fragments. Similarly, Greulich and Bornette (1999) found that highest production of shoots occurred before July. Altogether this indicates adaptation for dispersal during spring and colonization during autumn.

Based on the current knowledge colonization by *L. natans* shoots could occur as follows: at the end of summer or beginning of autumn the weather changes towards stronger winds and heavy rainfall. This combination will detach a lot of the younger shoots of *L. natans* and disperse them downstream in rivers or upon lakeshores. At the same time the weather could create new habitats by scouring the existent vegetation at the banks or shores. If a shoot were able to arrive at the site it would have the optimal conditions for attachment. This, however, has to happen before the next heavy rainfall. As the autumn proceeds the rain will increase the water level and the shoot will become submerged. Due to the fact that *L. natans* is evergreen (Greulich and Bornette, 1999, 2003) the shoot can slowly colonize the surrounding area during winter. In the next spring there could be a new population of *L. natans*, which, if it reaches the right depth and is able to compete with other colonizers, will be able to survive over time.

4.2. Dispersal and germination of seeds

Due to the combination of a large seed production, high abundance in the sediment and high germination rate, seeds seem to be an important factor in dispersal and colonization. A germination rate of 51–60% measured in the laboratory shows that most seeds were viable and able to germinate in situ. The seed bank dynamics in Kimmelkær Canal, which showed a higher abundance of seeds in the sediment before spring germination than after germination, support this. Also the genetic analysis indicates high dispersal by sexual reproduction as all samples could be differentiated, and, should, hence, originate from different individuals. The low sample sizes and long distance (1.5–5 m) between samples within populations reduces the chance of collecting more samples of the same individual. Therefore, vegetative reproduction by runners may be important in small scale within a population, but the results clearly document that the populations are polyclonal.

4.3. Vegetative or sexual reproduction

The extent to which *L. natans* reproduce by seeds or vegetative reproduction is highly dependent on type of disturbance at the site. The highest genetic diversity within location and population was found in Kimmelkær Canal subjected to management by dredging. Dredging removes most of the biomass of both *L. natans* and other species, and new populations have the opportunity to be founded by seeds. This is supported by earlier studies that indicate that viable seeds in the seed bank play an important role in recolonization after disturbances (Westcott et al., 1996). Furthermore, Willby et al. (2001) describes *L. natans* as a facultative ruderal in some habitats, including habitats subjected to dredging. They found large pure stands of *L. natans* in canals that were subjected to management by dredging.

The lowest overall genetic diversity was found in Lake Husby and River Skjern probably due to lack of flowering. In Lake Husby production of flowers was not observed in 2003 or 2004, and the population seems to be decreasing. High water velocity prevents flowering in River Skjern and, hence, lowers the genetic diversity at this location. Southern Parallel Canal and Gødelen are both managed by weed cutting, which results in low genetic diversity due to lowered production of flowers. Overall weed cutting and high water velocities result in high vegetative reproduction and dredging results in high sexual reproduction.

4.4. Distribution of L. natans in relation to dispersal

L. natans is usually only found in a small number of rivers and lakes within a region, but when it is found it usually occurs in high densities. This distribution pattern could have three origins: it is seen for specialists on rare resources, it is seen when species have been subjected to a decrease in habitats and it is seen for species with poor regional dispersal ability (Lansdown and Wade, 2003). When this distribution pattern is compared with the current knowledge of the ecology of *L. natans* the first explanation can be ruled out since *L. natans* is not considered to be dependent on specific resources. *L. natans* has to some extent been subjected to a decrease in habitats. All over its distribution area *L. natans* seems to be disappearing from oligotrophic lakes due to eutrophication and acidification, but at the same time the species appears in new habitats. However, it is worth emphasising that *L. natans* never has been widely distributed and in most cases suitable habitats are not occupied, so its high local abundance is not a relict of a former distribution pattern.

Most of the current knowledge of *L. natans* indicates that low dispersal ability between systems causes the distribution pattern. The absence in nearby lakes and rivers indicate that over ground dispersal can be a limitation for colonization of new habitats. Vegetative shoots are vulnerable to desiccation and would probably not maintain viability after much time in the air. Over ground dispersal would be expected to occur only by transportation of seeds. There are no exact measures of over ground dispersal by *L. natans* and it seems to be of little importance, but a find in Norway (Halvorsen and Grøstad, 2002) and a find in Sweden (Frits, 1989), both over great distances, indicates some dispersal by birds.

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