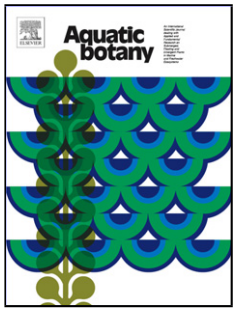


# Genetic diversity of *Nuphar lutea* in Lithuanian river populations

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# Genetic diversity of *Nuphar lutea* in Lithuanian river populations

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## Highlights

- Lithuanian populations of *Nuphar lutea* are genetically differentiated
- Reproduction is primarily sexual, and inbreeding appears to be common
- Genetic isolation and inbreeding could increase sensitivity to anthropogenic change

## ABSTRACT

Currently, in Europe, increasing attention is being paid to the genetic diversity of aquatic macrophytes. Insufficient information exists about the river plants of the Baltic states. Our study aimed to evaluate the genetic diversity of *Nuphar lutea* individuals growing in Lithuanian watercourses. Eighteen populations were studied in the river catchments of Lithuania: Nemunas, Venta and Lielupė. The genetic diversity of the populations was evaluated at microsatellite loci. The

population genetic data of *N. lutea* were analysed by multiple tests, including hierarchical analysis of molecular variance (AMOVA), principal coordinate analysis (PCoA) and the Mantel test. The observed ($H_O$) and expected ($H_E$) heterozygosity values per population were 0.242 - 0.655 and 0.503 - 0.759, respectively. Our study revealed significant differentiation among populations ($F_{ST}$ = 0.162; $p < 0.001$) and no correlation between genetic and geographical distances; this outcome is in agreement with relatively high inbreeding in populations and implies limited gene flow among subcatchments.

**Keywords:** simple sequence repeat (SSR) markers, aquatic ecosystems, aquatic plants, Nymphaeaceae

## 1. Introduction

Submerged macrophytes are important elements of river ecosystems that crucially contribute to proper ecosystem functioning and ensure effective ecosystem services. However, over the last 100 years, aquatic ecosystems have faced unprecedented challenges. Human-related activities in water bodies, such as river regulations, alter the hydrological regime of catchments, eventually resulting in changes in biodiversity (Fuller et al., 2015; Anderson et al., 2018). Most anthropogenic interventions are changing the quality and integrity of aquatic plant habitats. To assess the human impact on water ecosystems, manage water bioresources and assess species evolutionary potential, monitoring systems have been created (Schaumburg et al., 2004; Bradley et al., 2013). Among aquatic organisms, macrophytes are valuable indicator species due to their sedentary habit and visibility.

In recent decades, monitoring genetic diversity at the ecosystem and species levels has become a new sensitive tool for molecular genetics (Selkoe et al., 2006; Schwartz et al., 2007). Molecular techniques allow the measurement of changes in population genetic structure to assess

environmental variables related to the genetic divergence of populations. Fragmentation of habitats results in a decrease in genetic diversity and accelerates genetic drift. In small populations, inbreeding and genetic drift are enhanced and result in increased population differentiation. Various molecular techniques have been used to assess the genetic diversity of riparian plant species. Microsatellite (SSR) markers are among the preferred methods because of their reliability, codominant inheritance and high variability (Selkoe et al., 2006).

One perennially submerged macrophyte that is widespread in the temperate zone of North America and Eurasia is the yellow water lily (*Nuphar lutea* (L.) Sm.), which grows in shallow, slowly flowing water or standing water (Padgett, 2007). It is a diploid species (2n = 38) that is pollinated by bees and flies (Ervik et al., 1995), with frequent reproduction by rhizomes (Padgett, 2007; Fér and Hroudova 2008). Microsatellite markers have been developed for *N. lutea*, and polymorphisms at SSR loci have been studied in Polish (within Oder), Dutch (Rhine), French (Rhône) (Ouborg et al., 2000;) and Czech (Fér and Hroudova, 2008) populations.

We selected *N. lutea* due to its ecological relevance and wide distribution within Lithuanian water bodies. The natural network of Lithuanian rivers has changed significantly due to the regulation and draining of wetlands, especially within the second half of the previous century. Only approximately 17 % of riverbeds remain natural (Jablonskis et al., 2007). The aim of our research was to quantify the genetic diversity and genetic differentiation within and among populations of *N. lutea* growing in Lithuanian rivers that have undergone major changes over the past century.

## 2. Materials and methods

### 2.1. Population data and DNA assays

Populations of *N. lutea* were sampled from 9 Lithuanian river subcatchments, mainly belonging to the largest river catchment, Nemunas (Table S1). Following the *N. lutea* study, described by Fér and Hroudova (2008), which confirmed the dispersal of seeds and clones over tens

of kilometres, we collected plants at a distance of 100 m to more accurately sample the genetic diversity of populations and to avoid clones of identical genotypes. Clusters of leaf rosettes growing apart were considered separate individuals. The number of samples collected per site depended on the size of the site. Plant material for analysis was collected from 2 - 13 plants per site. Thus, the sampling site was specified as the assembly of plants growing along the river section for a few hundred metres until reaching 2 km. The distance between sites in the same river ranged from 6 to 53 km. In total, 37 sampling sites were examined (Table S1) during the summer of 2016.

Genomic DNA was extracted from 100 mg of fresh, healthy and cleaned (by distilled water) leaf tissue, that had been pulverised in liquid nitrogen. A Genomic DNA Purification Kit K0512 (Thermo Fisher Scientific Baltics, Lithuania) was used for DNA extraction. Six SSR loci were analysed following the descriptions of Ouborg et al. (2000) and Fér and Hroudova (2008). PCR was performed in a 10 µL total reaction volume containing 10 ng of DNA; 1 µL (10 pmol/µl) of the forward primer labelled with fluorophore NLGA2 - 6 FAM, NLGA3 - NED, NLGA5 - VIC, NLGA8 - NED, NLCA1 - 6FAM, and NLTG/GA1 - VIC; 1 µL (10 pmol/µl) of reverse primer; 5 µL of Dream Taq PCR Master Mix (Thermo Fisher Scientific Baltics, Lithuania) and 1 µL $H_2O$. PCR was performed under the following conditions: 1 min at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 50°C, 35 s at 72°C; and a final elongation step for 15 min at 72°C.

One microlitre each of 2 primer pairs (for the NLGA3 and NLGA5 loci) or 4 primer pairs (for the NLGA2, NLGA8, NLCA1 and NLTG/GA1 loci) was used to create two pools of PCR products for genotyping. Precipitated DNA was resuspended in 10 µL of Hi-Di formamide (Applied Biosystems, Inc.) and mixed with custom size standard GeneScan™ 600 LIZ™ (Applied Biosystems, Inc.). Genotyping was performed using a 3500 Genetic Analyser (Applied Biosystems, Inc.). GeneMapper software version v4.1 (Applied Biosystems, Inc.) was used to score the genotypes.

2.2. Data analysis

To avoid the undesirable effects of small sample sizes on population genetic diversity analysis, some geographically close sampling sites (minimum distance: 5 km; maximum distance: 37 km) were assembled into groups that together with larger sites (10 or more individuals) were regarded as populations (Table S1). The genotyping errors were evaluated by Micro-Checker v2.2.3 (van Oosterhout et al., 2004) using 1000 randomisations and according Bonin et al. (2004). The rate of genotyping error assessed according to Bonin et al. (2004) among the three replicates of the 30 arbitrarily chosen consensus samples was 0.6 %. Non-reproducible alleles were identified in consensus samples and were eliminated from the analysis of all samples. Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested using Genepop v4.7.0 (Rousset, 2008). The total number of alleles (Na), allelic richness ($A_{R10}$) for the minimum number of individuals per population (10 plants), and inbreeding coefficient (Fis) were calculated by FSTAT v2.9.4 (Goudet, 2003). GenAlEx v6.5 (Peakall and Smouse, 2012) was used to calculate the number of alleles recorded in each population (A), to calculate the observed ($H_E$) and expected ($H_O$) heterozygosities and for principal coordinate analysis (PCoA). Arlequin v3.5.2.2 (Excoffier and Lischer, 2010) was used to assess the genetic structure of *N. lutea* populations and genetic differentiation ($F_{ST}$) between the 18 populations. To eliminate the impact of null alleles on $F_{ST}$, the excluding null alleles (ENA) correction method in FreeNA software (Chapuis and Estoup, 2007) was applied. The bootstrapped 95 % confidence intervals (CIs) of the global $F_{ST}$ ENA were calculated using 2000 replicates for the loci. AMOVA performed in Arlequin v3.5.2.2 was used to assess genetic variation between individuals, populations and subcatchments and the significance of these parameters. The Mantel test implemented in GenAlEx v6.5 was performed with 9999 random permutations to assess the impact of geographical distance on population genetic structure, and correlations were calculated between genetic distance [(Fst/(1-Fst)] estimates for pairs of populations and pairwise geographic distances among populations.

## 3. Results and discussion

Our study of *N. lutea* populations revealed high clonal diversity, which is frequent in aquatic plants (Yakimowski and Barrett, 2014; Abbasi et al., 2016). Among the 279 plants analysed at six microsatellite loci, 272 different genotypes of *N. lutea* were identified. Clones were recorded in five populations (NL02, NL06, NL07, NL13 and NL15). This finding validated the sampling strategy and identified separate individuals. Other seven pairs of genotypes differed in only one allele. In contrast to the finding of a previous study of *N. lutea* in three Czech river catchments (Fér and Hroudova, 2008), genetically identical individuals were found only within populations. This outcome might be explained by the reduced spread of clones and by different sampling strategies. The number of detected alleles per population ranged from 21 - 52 (Table 1). Micro-Checker detected possible evidence of null alleles at all loci. The frequency of null alleles ranged from 0.067 to 0.232. Significant deviations from HWE were documented for all populations. No significant LD was observed between loci. Comparison of observed heterozygosities ($H_O$) revealed rather high variation in this parameter among populations, ranging from 0.242 (NL05) to 0.655 (NL15). In all cases, the $H_O$ values were lower than the expected heterozygosity ($H_E$). The lowest $H_E$ value was 0.503 (NL02), and the highest was 0.759 (NL03). The mean number of alleles (Na) per locus per population varied between 3.5 (NL05) and 8.667 (NL07). However, when the number of plants per population was rarefied to ten, the smallest number of alleles was 3.438 (NL04), and the largest was 7.221 (NL18). The average values of $H_E$ and $H_O$ for all the Lithuanian populations were 0.649 and 0.423, respectively. The $H_E$ value was similar to the average $H_E$ for long-lived perennials (0.68) (Nybom, 2004). The level of $H_E$ from our study was higher than that calculated for Czech populations of *N. lutea* ($H_E$ = 0.505) (Fér and Hroudova, 2008), possibly because of the slightly larger mean number of individuals studied per population in our study (Nybom, 2004). Similar results were obtained in other studies of aquatic plants with sexual and vegetative reproduction. For example, Abbasi et al. (2016), in a study of Iranian *Potamogeton pectinatus* from 36 sites, analysed

nine microsatellite loci and revealed averages of $H_E$ = 0.55 and $H_O$ = 0.54. When compared to our *N. lutea* data, the $H_E$ and $H_O$ values in the endangered species *Nuphar submerse* are significantly lower ranging from 0.10 to 0.24 and from 0.11 to 0.33, respectively (Shiga et al., 2017).

In our study, all populations had an $H_O$ that was lower than the expected heterozygosity, which may indicate a deficit of heterozygotic genotypes. The deficiency of heterozygotes may be explained by various factors, including non-random mating and the presence of null alleles (Sun and Salomon, 2003). We also revealed relatively high values of the inbreeding coefficient (Fis) for many populations. The highest Fis (0.614) was documented for the NL10 population, and the lowest inbreeding coefficient (0.196) was documented for the (NL06) population. Fis values, calculated per population, indicate that deviation from HWE could be related to inbreeding. In agreement with the results documented for the same species growing in more southern or western parts of Europe, the probability of inbreeding events is realistic for Lithuanian *N. lutea* populations, and in general, this phenomenon is consistent with the biology of *N. lutea* (Padgett 2007). Although *N. lutea* is protogynous and mainly cross-pollinated, this species is also self-compatible (Ervik et al., 1995). The possibility of fertilisation by self-pollination has been demonstrated by transferring pollen grains to the stigma of the same flower (Ervik et al., 1995; Lippok and Renner 1997).

The average genetic differentiation ($F_{ST}$) among *N. lutea* populations was high (0.162; $p < 0.001$). The highest value of $F_{ST}$ was between the NL05 and NL14 populations (0.235), and the smallest difference was between populations NL16 and NL17 (0.026). The $F_{ST}$ estimated using the ENA correction was lower ($F_{ST}$ = 0.141) (CI 95 %, 0.111 - 0.173). According to the hierarchical AMOVA, 7 % of the genetic variation was partitioned among the subcatchments ($p < 0.001$). Variation among the populations inside the subcatchments totalled 13 % ($p < 0.001$). The remaining variability was partitioned within the populations ($p < 0.001$). The value of $F_{ST}$ obtained here differs to some extent from the values obtained for Czech populations (Fér and Hroudova 2008), where approximately 22 % of the genetic variability was found among the sections of the rivers. The

possible reason for this difference can be accounted for by the smaller number of loci analysed in our study or some historical and ecological factors that may differ across species ranges (Yakimowski and Eckert, 2008).

The first and second axes of the PCoA explained 54.8 % of the entire variation. The PCoA plot exhibited only a weak grouping of populations according to their geographic locations (Fig. 1). One cluster formed four geographically related populations in the Nevėžis subcatchment (NL15-NL18). However, most populations were scattered independently of their geographic locations. These results of PCoA are in agreement with the results of the Mantel test, which did not indicate a correlation between genetic and geographical distances at the level of the total set of sampling sites ($R^2 = 0.014$, $p = 0.1$) or at the level of the Nemunas River catchment ($R^2 = 0.011$, $p = 0.08$). No correlation was detected along different transects starting in the upper reaches of different rivers and ending in the lower reaches of the Nemunas River. The first reason might be isolation of river subcatchments included in the study and the increased self-pollination rate, indicating that spatial genetic structure was not caused by gene flow among geographically close populations. Presumably, some factors of anthropogenic origin might also be involved. The Lithuanian rivers have changed significantly due to regulation and straightening (Jablonskis et al., 2007). An impact of anthropogenic disturbance on genetic diversity patterns among the other plant species has been reported in other surveys. For example, Anderson et al. (2018) revealed that the number of polymorphic SSR loci was larger for populations of *Phalaris arundinacea* on the banks of the natural stretches of the river Merkys (Lithuania) than for those in the regulated stretches. Aparicio et al. (2012) pointed out that the response of species to anthropogenic impacts takes various forms, and that the direction of influence on genetic structure may vary. For instance, the pattern of genetic diversity of populations of *Phragmites australis* showed little correlation with the degree of human impact on the wetlands, although populations that experienced a lower level of disturbance were less genetically diverse (Lambertini et al., 2008). Additionally, it cannot be ruled out that the

genetic structure of Lithuanian populations of *N. lutea* is impacted by climatic or edaphic factors or both.

In conclusion, information on the extent of genetic diversity and differentiation within and between Lithuanian populations of *N. lutea* may aid in the future monitoring of the species against the backdrop of continuous anthropogenic impact and climate warming because isolated inbred populations may be more susceptible than other populations to anthropogenic and environmental impacts.

**Declaration of interest**

No conflicts of interest.

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**CRediT authorship contribution statement**

**Donatas Žvingila**: Conceptualization, Funding acquisition, Formal analysis,Writing – review & editing. **Regina Vyšniauskienė**: Data curation, Investigation, Formal analysis. **Vida Rančelienė**: Data curation, Investigation, Formal analysis. **Donatas Naugžemys**: Software, Formal analysis, Methodology. **Eglė Rudaitytė-Lukošienė**: Formal analysis, Methodology. **Jolanta Patamsytė**: Formal analysis, Software, Visualization. **Dalius Butkauskas**: Resources, Methodology. **Eugenija Kupčinskienė**: Writing – review & editing.

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**Figure legend**

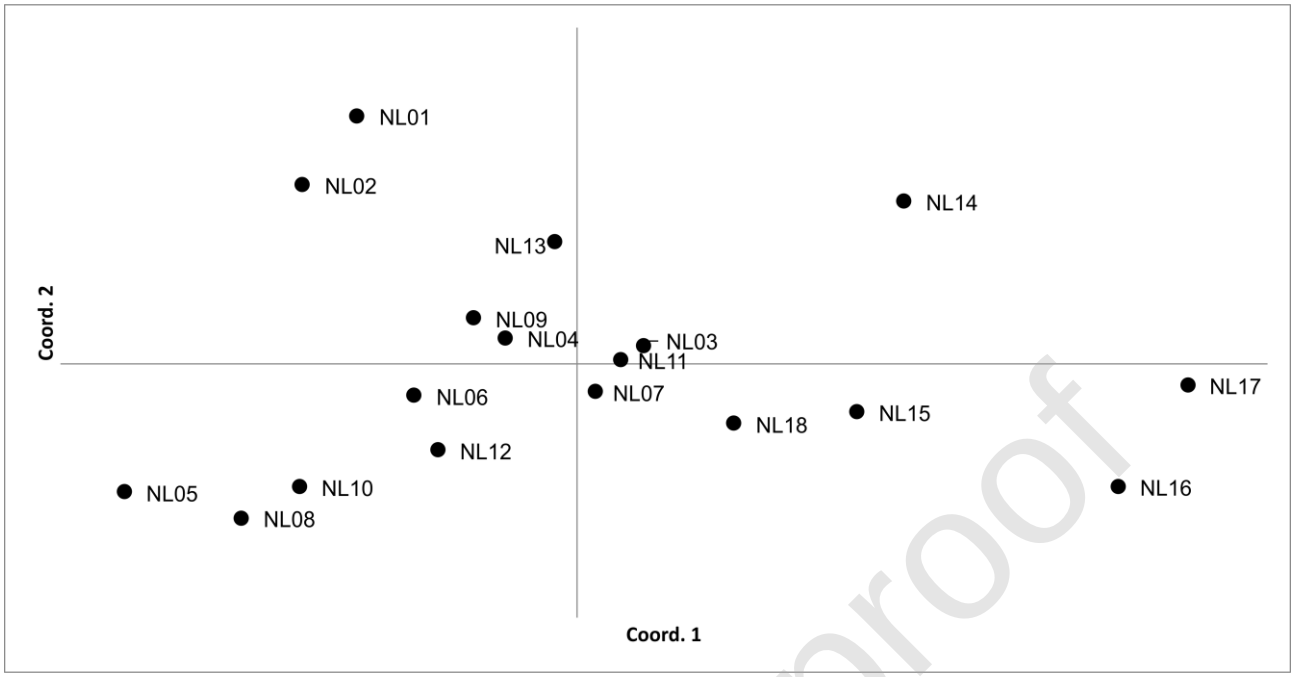


**Fig. 1.** SSR loci-based principal coordinate analysis of genetic relationships among Lithuanian populations of *Nuphar lutea*.

**Table 1**: Genetic diversity parameters (number of different genotypes in population – n, number of alleles recorded in each population – A, number of alleles – Na, average allelic richness based on 10 genotypes –$A_{R10}$, inbreeding coefficient – Fis, observed heterozygosity – $H_O$, expected heterozygosity – $H_E$) of Lithuanian populations of *Nuphar lutea*

| Popcode | n | A | Na | $A_{R10}$ | Fis | $H_O$ | $H_E$ |
|---|---|---|---|---|---|---|---|
| NL01 | 10 | 25 | 4.167 | 3.603 | 0.345 | 0.417 | 0.593 |
| NL02 | 11 | 22 | 3.667 | 6.287 | 0.408 | 0.318 | 0.503 |
| NL03 | 23 | 49 | 8.167 | 5.358 | 0.389 | 0.478 | 0.759 |
| NL04 | 13 | 35 | 5.833 | 3.438 | 0.410 | 0.397 | 0.637 |
| NL05 | 11 | 21 | 3.500 | 4.816 | 0.600 | 0.242 | 0.562 |
| NL06 | 11 | 30 | 5.000 | 6.754 | 0.196 | 0.530 | 0.624 |
| NL07 | 21 | 52 | 8.667 | 4.127 | 0.426 | 0.444 | 0.748 |
| NL08 | 14 | 27 | 4.500 | 5.383 | 0.340 | 0.405 | 0.583 |
| NL09 | 21 | 38 | 6.333 | 4.850 | 0.363 | 0.476 | 0.723 |
| NL10 | 13 | 32 | 5.333 | 4.978 | 0.614 | 0.269 | 0.653 |
| NL11 | 16 | 33 | 5.500 | 6.000 | 0.316 | 0.500 | 0.701 |
| NL12 | 10 | 36 | 6.000 | 5.026 | 0.304 | 0.517 | 0.694 |
| NL13 | 11 | 31 | 5.167 | 4.619 | 0.395 | 0.409 | 0.633 |
| NL14 | 23 | 34 | 5.667 | 6.060 | 0.310 | 0.413 | 0.582 |
| NL15 | 14 | 40 | 6.667 | 5.093 | 0.304 | 0.655 | 0.722 |
| NL16 | 14 | 34 | 5.667 | 5.383 | 0.425 | 0.381 | 0.629 |
| NL17 | 21 | 41 | 6.833 | 5.132 | 0.466 | 0.349 | 0.631 |
| NL18 | 15 | 35 | 5.833 | 7.221 | 0.427 | 0.422 | 0.702 |
| Mean | | 34.17 | 5.695 | 5.229 | 0.391 | 0.423 | 0.649 |
| SE | | 1.92 | 0.320 | 0.233 | 0.024 | 0.023 | 0.017 |

* SE – standard error