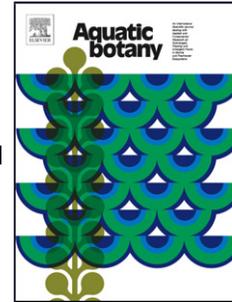


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Preliminary insights into genetic diversity and history of *Nuphar lutea* and *N. pumila* (Nymphaeaceae) in isolated southwestern European populations

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Running title: *Nuphar* in southwestern Europe

Highlights

- We investigate genetic variability of isolated populations of *Nuphar lutea* and *N. pumila*.
- We sequenced nuclear ribosomal ITS and chloroplast intergenic spacers.
- No evidence for southwestern European long-term refugium for *Nuphar* species.
- *Nuphar pumila* and *N. lutea* spread from the Far East to Europe.
- No signs of hybridization among *Nuphar lutea* and *N. pumila* were found.

ABSTRACT

Recent phylogeographical study of Eurasian *Nuphar* species has revealed that after the subgenus diversified in eastern Asia, *N. pumila* and *N. lutea* extended westwards to Siberia and further into Europe. However, isolated *Nuphar* populations from southwestern Europe were not included in

previous phylogeographic study and their history remained unknown. Given the extreme rarity of *N. pumila* in southwestern Europe, the genetic study of degree and direction of introgression between this species and *N. lutea* in the region is highly warranted. We investigated the genetic variability of isolated populations of *N. lutea* and *N. pumila* from central France and northern Spain, using nuclear (ITS) and cpDNA sequences. We identified species-specific haplotypes and ribotypes for *N. pumila* and *N. lutea*. We found no evidence for southwestern European refugia for *N. lutea* and *N. pumila* and rather support the hypothesis of their migration from the East. We found no signs of hybridization between *N. lutea* and *N. pumila*. However, our results should be verified with more sensitive molecular markers.

Keywords: cpDNA; hybridization; isolated populations; ITS; phylogeography.

1. Introduction

Nuphar Sm. (Nymphaeaceae), the yellow water-lilies, are a small group of perennial hydrophytes that primarily inhabit temperate fresh waters. Only two species in the genus (*N. pumila* (Timm.) DC. and *N. lutea* L.) naturally occur in Europe along with their fertile hybrid *N. × spenneriana* Gaudin (Padgett, 2007). While *N. lutea* is widely spread in the region, the climate relict *N. pumila* is rare in central and western Europe, present in lowlands and peaty lakes, and sometimes, being restricted to very scattered mountain lakes (Arrigo et al., 2016; Bétrisey et al., 2020). Recent phylogeographical study of Eurasian *Nuphar* species revealed that after the subgenus diversified in eastern Asia *N. pumila* and *N. lutea* extended westwards to Siberia and further into Europe (Volkova et al., 2018). However, *Nuphar* populations from the southwestern Europe where important glacial refugia were reported (Hewitt, 1999) were not included in that study and their history remains unknown.

Recent genetic studies of yellow water-lilies in Alpine arc region demonstrated ongoing bidirectional introgression between *N. lutea* and *N. pumila*, causing extinction risk for specialist *N. pumila* (Arrigo et al., 2016; Bétrisey et al., 2020). However, the opposite situation was revealed in

the British Isles: *N. × spenneriana* displaced *N. lutea* at its more northerly locations because of its competitive advantage in tolerating cooler water (Vallejo-Martin and Hiscock, 2016). Additionally *N. pumila* and *N. lutea* hybridize rarely in eastern Europe and Asia even if they are co-occurring in one waterbody (Volkova et al., 2018). Given the extreme rarity of *N. pumila* in the southwestern Europe (Fernández Bernardo De Quirós, 1983; Arrigo et al., 2016), the genetic study of degree and direction of introgression between this species and *N. lutea* in the region is highly relevant.

In this study, we investigate genetic variability of isolated populations of *N. lutea* and *N. pumila* from southwestern Europe, using nuclear (ITS) and cpDNA sequences to (1) deduce their history in the context of the previous area-wide phylogeographic study (Volkova et al., 2018) and (2) evaluate the degree and direction of hybridization between these two species.

2. Material and methods

2.1. Plant material and DNA extraction

Fresh leaves of *N. lutea* and *N. pumila* were sampled from four localities in Spain and France, representing the fragmented and isolated populations of southwestern Europe (Table 1, Fig. 1). Voucher specimens are kept in the Herbarium of the University of Oviedo (FCO). Samples for molecular analyses were dried in silica gel and stored at room temperature prior to DNA isolation. DNA was extracted using the DNeasy Plant Mini Kit system (Qiagen), according to the protocol recommended by the manufacturer. DNA concentration was measured by a Beckman-Coulter DU800® spectrophotometer (Fullerton, CA, USA).

2.2. DNA amplification and sequencing

PCR reactions were performed following Cires et al. (2018). Standard primers were used for amplification and sequencing of complete ITS (White et al., 1990) and plastid regions, analyzed by Volkova et al. (2018): *trnL-trnF* (Taberlet et al., 1991) and *trnH-psbA* (Shaw et al., 2005). PCR products were sequenced in both directions at the DNA Synthesis and Sequencing Facility Macrogen (Madrid, Spain). Sequence data were assembled using ClustalW and edited with

Geneious 7 (Kearse et al., 2012). The haplotype diversity of the data was verified using DNASP6 v.6.12.01 (Rozas et al., 2017). The obtained sequences are deposited in GenBank (see Table 1 for accession numbers). We also used the sequences of *N. advena* (Aiton) W.T.Aiton and *N. variegata* Engelm. from GenBank as an outgroup (following Padgett, 2007) and included for comparison sequences of *N. pumila* subsp. *sinensis* (Hand.-Mazz.) D. Padgett (*N. sinensis* Hand.-Mazz.), retrieved from GenBank, and additionally sequences of *N. pumila* and *N. lutea*, published by Volkova et al. (2018): Table S1

2.3. Phylogenetic analyses

Phylogenetic analyses of nuclear and plastid DNA were performed using Maximum Parsimony (MP) and Maximum Likelihood (ML) methods and conducted by a heuristic search using MEGA 7.0 (Kumar et al., 2016). The MP consensus tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm with search level 3, in which the initial trees were obtained by random addition of sequences (100 replicates). On the other hand, ML consensus tree was inferred using Kimura 2-parameter model (Kimura, 1980). In both phylogenetic approaches, the bootstrap consensus tree was inferred from 1000 replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Relationships among haplotypes and ribotypes were inferred using the statistical parsimony method implemented in TCS software (Clement et al., 2000) and gaps were treated as the fifth state.

3. RESULTS AND DISCUSSION

All the analyzed *N. pumila* samples from the two localities from southwestern Europe were characterized by haplotype d and ribotype C (Figs. 1, S1 and S2). This genotype was also found in all *N. pumila* plants across eastern Europe and western Siberia and the only analyzed locality from central Europe (Volkova et al., 2018). Almost all the analyzed southwestern European *N. lutea* had haplotype b and ribotype A (Figs. 1, S1 and S2). The only exception was one plant from Spain with the ribotype A**, which is a close derivative of A, differing from it by one position in the length of

poly-C region (Table S1). This genotype (b/A) was confined to *N. lutea* from central and eastern Europe (Volkova et al., 2018); it was erroneously reported for *N. japonica* DC. from the Russian Far East by Volkova et al. (2018) due to analytical error (P.A. Volkova, unpubl.). Low genetic variability of *N. pumila* subsp. *pumila* (one ribotype and two haplotypes, separated by the Lena river) has been already shown for the main part of its area (Volkova et al., 2018). However, low genetic variability of *N. lutea* that we revealed in southwestern Europe is in contrast with the usual coexistence of several haplotypes in other parts of its range (Volkova et al., 2018). Thus, we found no evidence for southwestern European long-term refugium for *Nuphar* species and rather supported hypothesis of their migration after the glaciations from the East (Volkova et al., 2018). Our results disagree with the treatment of alpine *N. pumila* as a "long-term store of genetic diversity" (Bétrisey et al., 2020). The verification of the assessment made by Bétrisey et al. (2020) would require an area-wide study of microsatellite variation with an increased number of sampled locations of western European *N. pumila*. Moreover, the evolutionary history of *N. lutea* remains unclear, due to the complex structure of its genetic variability on the area-wide scale (e.g. one haplotype shared with *N. pumila*: Volkova et al., 2018).

We found no signs of hybridization between *N. lutea* and *N. pumila* (i.e. additive polymorphic sites of ITS, combining the respective positions of the two species that were reported for eastern Europe: Volkova et al., 2018). This is at odds with high levels of introgression between *N. lutea* and *N. pumila* found in other regions of western Europe (Arrigo et al., 2016; Vallejo-Martin and Hiscock, 2016). However, although our data provided no evidence for hybridization, we cannot rule out the possibility its presence (if e.g. ITS is homogenized due to the concerted evolution towards the ribotype of one parent). In this respect, more populations should be explored with more sensitive molecular markers (e.g. microsatellites: Ouborg et al., 2000; Arrigo et al., 2016; Bétrisey et al., 2020).

Over the past 15 years, attention to plant conservation problems in aquatic ecosystems has increased in western Europe (Bañares et al., 2004; Moreno, 2008; Lansdown, 2017; O'Hare et al., 2018).

Notably, knowledge of aquatic plants on the Iberian Peninsula has increased in recent years, leading to emergence of conservation strategies as well as fighting against the main threats of protected plants linked to water (Cirujano Bracamonte and García Murillo, 2019; Vallejo Pedregal et al., 2019). This is the case for the Spanish population of *N. pumila*, which deserves special interest because it is separated by about 800 km from the nearest populations in the French Massif Central. Its threat assessment (Bañares et al., 2004; Moreno, 2008) led to the categorization of this plant as "vulnerable" (VU, application of the D1+2 criteria from International Union for Conservation of Nature). At present and based on the results obtained, no more potential threats have been detected for the Spanish population than a possible decrease in the water level, forestry clearance or increased supply of humus compounds. Similar situations have been reported in other isolated populations of *N. pumila* in central Europe (Dynowski et al., 2016; Bétrisey et al., 2020).

AUTHOR STATEMENT

All analyses and figures are new, and the manuscript has not been submitted to any other journal and the authors reported no potential conflict of interest.

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Fig. 1

Geographical locations and genetic differentiation of ITS ribotypes (A-B) and cpDNA haplotypes (C-D) of *Nuphar lutea* and *N. pumila* obtained in this study.

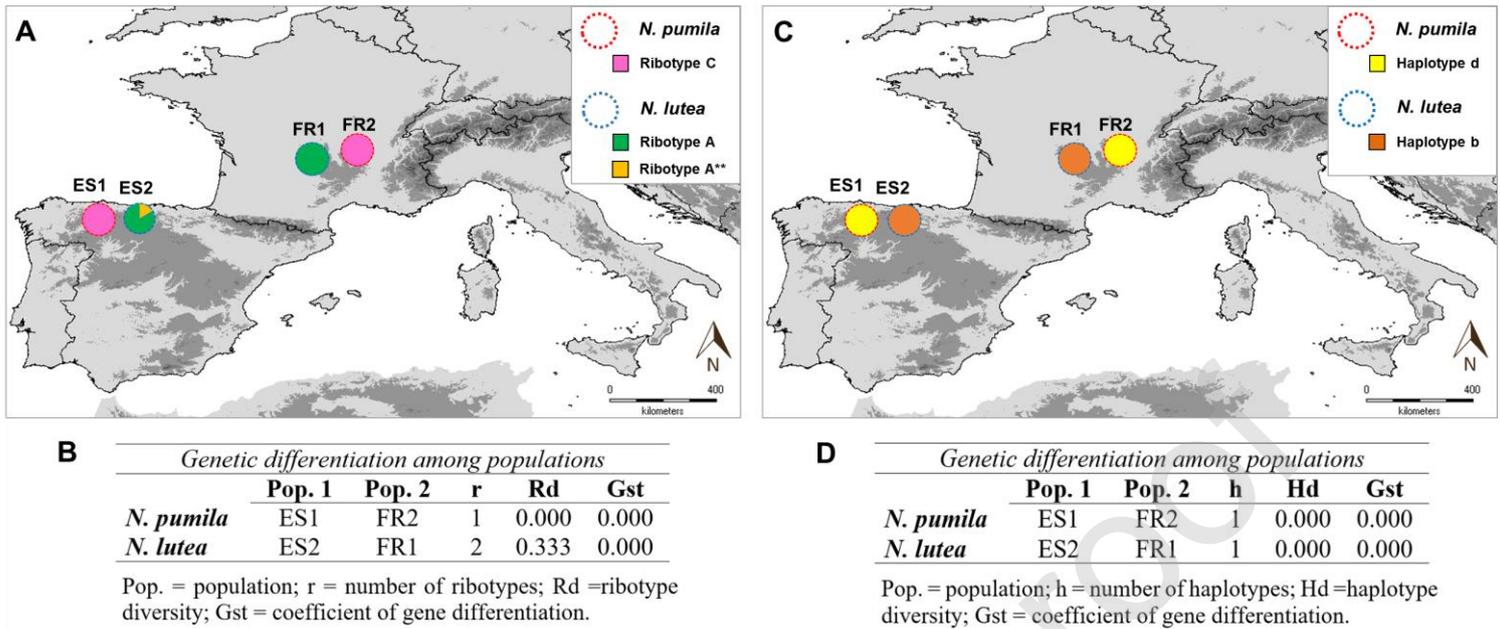


Table 1

Population codes, localities and GenBank accessions for DNA sequences of *Nuphar pumila* and *N. lutea* analyzed in the present study.

Code	Population / Coordinates / Herbarium voucher number	GenBank accession number		
		ITS	<i>trnL-F</i>	<i>trnH-psbA</i>
ES1	“Laguna de Reconcos, Principado de Asturias, Spain” / 43°0'1.50"N, 6°30' 35.61"W / FCO-39242		ES1-1 (MT300339)	ES1-1 (MT300366)
			ES1-2 (MT300340)	ES1-2 (MT300367)
		ES1-1 (MT300327)	ES1-3 (MT300341)	ES1-3 (MT300368)
		ES1-2 (MT300328)	ES1-4 (MT300342)	ES1-4 (MT300369)
		ES1-3 (MT300329)	ES1-5 (MT300343)	ES1-5 (MT300370)
			ES1-6 (MT300344)	ES1-6 (MT300371)
			ES1-7 (MT300345)	ES1-7 (MT300372)
			ES1-8 (MT300346)	ES1-8 (MT300373)
ES2	“Río Camesa, Mataporquera, Cantabria, Spain” / 42°52'20.73"N, 4°9'46.81"W / FCO-39243, 39244	ES2-1 (MT300330)	ES2-1 (MT300347)	ES2-1 (MT300374)
		ES2-2 (MT300331)	ES2-2 (MT300348)	ES2-2 (MT300375)
		ES2-3 (MT300332)	ES2-3 (MT300349)	ES2-3 (MT300376)
FR1	“Lac de Laspialade, France” / 45°24'59.68"N, 2°41'41.72"E / FCO-39245	FR1-1 (MT300333)	FR1-1 (MT300350)	FR1-1 (MT300377)
		FR1-2 (MT300334)	FR1-2 (MT300351)	FR1-2 (MT300378)
		FR1-3 (MT300335)	FR1-3 (MT300352)	FR1-3 (MT300379)
			FR1-4 (MT300353)	FR1-4 (MT300380)
			FR1-5 (MT300354)	FR1-5 (MT300381)
			FR1-6 (MT300355)	FR1-6 (MT300382)
			FR1-7 (MT300356)	FR1-7 (MT300383)
			FR1-8 (MT300357)	FR1-8 (MT300384)
FR2	“Lac de La Landie, France” / 45°25'8.29"N, 2°45'41.44"E / FCO-39246, 39247	FR2-1 (MT300336)	FR2-1 (MT300358)	FR2-1 (MT300385)
		FR2-2 (MT300337)	FR2-2 (MT300359)	FR2-2 (MT300386)
		FR2-3 (MT300338)	FR2-3 (MT300360)	FR2-3 (MT300387)
			FR2-4 (MT300361)	FR2-4 (MT300388)
			FR2-5 (MT300362)	FR2-5 (MT300389)
			FR2-6 (MT300363)	FR2-6 (MT300390)
			FR2-7 (MT300364)	FR2-7 (MT300391)
			FR2-8 (MT300365)	FR2-8 (MT300392)