Short communication

Is *Nymphaea lotus* var. *thermalis* a Tertiary relict in Europe?

Levente Laczkó[a,1], Balázs András Lukács[b,1*], Attila Mesterházy[c], Attila Molnár V.[a], Gábor Sramkó[a,d]

[a] Department of Botany, University of Debrecen, H-4032, Debrecen, Egyetem tér 1., Hungary

[b] Department of Tisza-River research, MTA Centre for Ecological Research, H-4026 Debrecen, Bem tér. 18/C, Hungary

[c] Hortobágy National Park Directorate, H-4026 Debrecen Sumen u. 2., Hungary

[d] MTA-DE "Lendület" Evolutionary Phylogenomics Research Group, H-4032 Debrecen, Egyetem tér 1, Hungary

* Corresponding author.

E-mail address: lukacs.balazs@okologia.mta.hu

[1] Contributed equally to this work.

Abstract

Nymphaea lotus L. is a tropical and subtropical species of waterlilies with an African distribution. A conspicuous satellite occurrence can be found in Europe in a thermal spring in NW Romania. This population (treated as var. *thermalis* (DC.) Tuzson) received much attention as a potential Tertiary relict in the flora of Europe, although its relict *vs*. planted nature has been part of great debate among scholars during the last 200 years. We revisit this question by using molecular phylogenetic methods to estimate the timing of divergence of this species, and put this population into a phylogeographic context by comparing it to samples coming from the whole area of the species. By using sequences of the nuclear and the plastid genome, we reconstructed the phylogeographic relationships within *N. lotus* with a haplotype network building approach, that identified a genetically distant lineage in western Africa, which we relate to *N. zenkeri* Gilg, the sister species of *N. lotus*. All the populations of *N. lotus* s.str. displayed genetic distance between each other except for the Egyptian and Romanian populations. On our dated phylogeny of the subgenus *Lotus* the separation between *N. lotus* and its sister species was found to be younger than the end of the Tertiary (1.26 Mya). These results clearly refute the Tertiary relict status of the European population of *N. lotus*.

***Keywords*:**

cultural relict; *Nymphaea* subg. *Lotos; N. zenkeri;* phylogeography; satellite population; Natura 2000, Transylvanian hot-spring lotus beds (31A0); thermal refugium

1. Introduction

The waterlily genus (*Nymphaea* L.) has five subgenera and includes mostly tropical aquatic plants. A subgenus of the tropical Old World is subg. *Lotos* D.C. with two species in Africa, three in Asia (Conard, 1905). An African member of the genus is *Nymphaea lotus* L., whereas its sister species (sometimes also referred to as *N. lotus* in a wide sense) is *N. pubescens* Willd. (Borsch et al., 2007), which grows in South-East Asia. There is a conspicuous satellite occurrence of *N. lotus* in Europe, in a thermal lake (Lake Peţea near to Oradea city, NW Romania), *ca* 3000 km away from the nearest location (the Nile Delta in Egypt). The European occurrence was found by Pál Kitaibel in 1798 during his trip to NE Hungary (the territory belonged to the Hungarian Kingdom in that period). One century later, several influential scholars started to advocate their opinion about the autochthonous nature of this population (e.g., Pax, 1905; Staub, 1903), thus, concluded the Tertiary relict origin of this species in the thermal lake. Literally speaking, this would mean the survival of this *Nymphaea* population in lake Peţea during the last *ca* 2.5 Myr including four main glaciations. Additional evidence for the Tertiary relict nature of the plant came from fossil leaves of *Nymphaea* species found in the region (Pax, 1905) and the co-occurrence of a freshwater snail from a predominantly (sub)tropical genus, *Melanopsis parreysii* (Phillippi R.A. 1847) (Brusina, 1902), together with the waterlily. A study based on pollen analysis also supported the relict status of this species (Diaconeasa and Popa, 1964). However, some scholars expressed their doubts about the relict status of this species and advocated its origin as result of recent dispersal by water birds (e.g., Borbás, 1894; Richter, 1897) or plantation by Turkish during the Ottoman occupation of Hungary in the 16^{th}–17^{th} century (Tuzson 1908). Despite of this evident uncertainty, it is generally accepted nowadays that the population in the thermal lake near Oradea, treated taxonomically as *Nymphaea lotus* L. var. *thermalis* (D.C.) Tuzson, is a Tertiary relict in the Romanian (Pop, 1976), and an indigenous member of the European Flora (Jalas and Suominen, 1989; Tutin and Webb, 1992). The conservation importance of the plant is reflected by the designation of an original habitat type of the Natura 2000 network, Transylvanian hot-spring lotus beds (31A0), to the preservation of the unique community at this location including the local *N. lotus* population.

Molecular phylogenetic and phylogeographic approaches together with the usage of the molecular clock make it possible to revisit the relict nature of this population. In light of the detailed phylogenetic information on the genus *Nymphaea* including the dated phylogeny of the genus (Borsch et al., 2007; Löhne et al., 2008), we attempt to investigate the speciation rates within subg. *Lotos* and the genetic distances within *Nymphaea lotus* using fast-evolving, neutral DNA regions from the nuclear and the plastid genome. If *Nymphaea lotus* var. *thermalis* is really a Tertiary relict taxon and the speciation rate in the subgenus is constant, a great genetic distance must exist between *Nymphaea lotus* subsp. *lotus* and the European population. Additionally, the divergence of

other species within the subgenus must pre-date the separation of the conspecific populations in Africa and Europe.

2. Materials and Methods

2.1 Sampling and laboratory protocols

Our field sampling represents the whole distribution area of *Nymphaea lotus* s.l. (Table S1). Samples were collected and stored in either 96% ethanol or in silica gel. These field samples were genotyped for four DNA regions: the nuclear ribosomal ITS (nrITS) and three plastid spacers (*rpl*32-*trn*L, *psb*M-*trn*D, *trn*T-*trn*L). Newly generated DNA-sequences were uploaded to GenBank (Table S3).

For our field collected samples, we extracted genomic DNA using a modified CTAB method as detailed here. 800 µl of extraction buffer consisting of 2% CTAB, 0.1 M Tris-HCl (pH=7.5), 1.4 M NaCl and 0.02 M EDTA were added to the ground leaves. After 1 hour of incubation on 65°C the mixtures were washed twice with chloroform : isoamylalcohol (24 : 1). DNA was precipitated using 100% isopropanol. After centrifuging pellets were washed twice with 70% ethanol, then air-dried and resuspended in 1 mM Tris-HCl (pH=7.5) buffer. Primer informations and their sequences used in the amplification and sequencing of the four loci involved in our study are listed in Table S2. Polymerase Chain Reactions (PCR) were conducted in 25 µl containing 1× Reaction Buffer (Thermo Scientific), 0.2 mM of each dNTP (Thermo Scientific), 25 mM $MgCl_2$, 0.25 mg Bovine Serum Albumin (Invitrogen), 0.2 µM of each primer, 0.03 U of Taq Polymerase (Thermo Scientific) and 100 ng DNA template. PCR amplicons were sequenced using a commercial service (Macrogen, Korea). PCR conditions for nrITS were the following: initial 3 min denaturation at 94°C followed by 35 cycles of denaturation for 30 sec at 94°C, 30 sec annealing at 51°C, 40 sec extension on 72°C and a final extension for 7 min at 72°C. For all the plastid markers we used the same touchdown PCR profile: initial denaturation for 3 min at 94°C followed by 20 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 58°C at the first cycle then lowering the temperature by 0.5°C in each cycle, then extension for 50 sec at 72°C. The same cycling conditions were repeated for another 20 cycles except that annealing temperature were kept at 48°C in every cycle, then final extension were conducted for 7 min at 72°C. Sequences were checked for errors and aligned by MUSCLE v.3.8.31 (Edgar, 2004). Accession numbers of sequences generated for the study can be found in Table S3.

2.2 Analysis of evolutionary rates and phylogeographic patterns

To assess evolutionary rates within *Nymphaea* subg. *Lotos*, we first assessed divergence times of taxa that are relatively closely related and diverged less than 25.4 Mya (Löhne et al. 2008). This analysis was based on the nrITS + *trn*T-*trn*L dataset and was run using *BEAST v.2.5.0 (Bouckaert et al., 2014). Sequences of species that were not available for us as field-collected samples were obtained from GenBank (Table S3). As our phylogeographic analyses (see below) unrevealed a significant genetic distance between western African samples and the rest in *N. lotus* s.l., we treated this population as a separate species (*Nymphaea zenkeri* Gilg) in the divergence time analyses. *BEAST was run two times for one billion generations using the uncorrelated lognormal relaxed clock model with a Yule process of speciation. Bayesian Model Test was run simultaneously with the data to find the optimal *a priori* molecular evolutionary model for the analysis. Runs were only accepted if Effective Sample Size (ESS) values were >200. The topology was constrained for the outgroup topology and secondary dating was accomplished by fixing the mean values of the chronogram of Löhne et al. (2008). Two calibration points were applied. We constrained the divergence of *Nymphaea* subg. *Nymphaea* from the rest of the taxa as the root age in our analysis (25.4±14.3 Mya). The second calibration point was fixed for the separation of *Nymphaea* subg. *Lotos* and *Nymphaea* subg. *Hydrocallis* (11.3±7.8 Mya). Bayesian analysis of macro-evolutionary mixtures (BAMM) (Rabosky, 2014) was run for 10 million generations sampling every 10000 generations on the reconstructed species tree with global sampling probability set to 0.6 (i.e., we suppose our sampling covers >60% of the species in the lineage). The first 10% was discarded as burn-in, then ESS was checked for convergence using the R package BAMMtools (Rabosky et al., 2014) which we also used for estimating evolutionary rates over time and most probable number of rate shifts.

To investigate the phylogeographic pattern and genetic distances within *Nymphaea lotus* s.l., we reconstructed the haplotype network of the field sampled populations. In addition to nrITS and *trn*T-*trn*L, this dataset was supplemented with two additional plastid DNA-regions (*rpl*32-*trn*L and *psb*M-*trn*D) to investigate phylogeographic relationships within this group in more detail. Haplotype reconstruction on the concatenated dataset (3523bp) was done in the R package PEGAS (Paradis, 2010). As an outgroup, we included the W African *Nymphaea zenkeri* population.

3. Results and Discussion

Our estimate of divergence times (Fig. 1) for the main lineages of *Nymphaea* and related genera fit in the 95% HPD interval described in Löhne et al. (2008). The lineage of subg. *Lotos* emerged 5.07 (95% HPD: 0–10.32) Mya, while the separation between *N. pubescens* and *N. lotus* is estimated to be 2.85 Myr old (95% HPD: 0–6.49), which correspond to the late Pliocene. Divergence between *N.*

lotus and *N. zenkeri* is assessed to have happened 1.26 Mya (95% HPD: 0–3.62) in Mid-Pleistocene. According to the results of BAMM, we detected no sign of significant rate shift in the phylogeny reconstructed based on the dataset, although a continuous decrease is visible (Fig. 1). Mean speciation rate on the whole chronogram varies between 0.17–0.26 species/Myr, within subg. *Lotos* mean rate varies between 0.17–0.18 species/Myr. If the European population was a Tertiary relict isolated at least *ca* 2.5 Mya, given the approximately constant rate of speciation in the lineage, we would expect it to be separated from the rest of the samples of *N. lotus* s.s. with a significant genetic difference between them, similar to what we see between *N. lotus* s.l. and *N. pubescens*. However, this is not the case as our phylogeographic analysis of *N. lotus* s.l. samples did not find a significant genetic distance between the African and the European plants (Fig. 2). Nonetheless, samples from the western African population showed remarkable divergence: five hypothetical haplotypes separated these plants from the rest, whereas we can only see single steps between the other samples of *N. lotus*. This separation of the western African plant might correspond to the major clades associated with the main refugial areas in Africa (Hewitt, 2004), and can validate the taxonomic opinion of those authors who separated the western African populations of *N. lotus* s.l. as *N. zenkeri* (see Verdcourt, 1989). The East African (Lake Ziwai) and the South African (Nylsvley) populations are only two steps (i.e., one hypothetical haplotype) away from the North African (Nile Delta) population. Surprisingly, the European population shares an identical DNA sequence on 3523bp length of rapidly evolving DNA regions with the plant from the Nile Delta. This stands in stark contrast to the alignment of nrITS sequences between *N. lotus* and *N. pubescens*, taxa that diverged before the Pleistocene (2.85 Mya), where we can find 27 polymorphic sites and two singleton indels on 645bp length (our sample 'lotEgy' aligned against GenBank accession FJ597743 of *N. pubescens*). Similarly, the alignment of *trn*T-*trn*L contains two SNP-s and two minisatellite indel motifs on 1504 bp length (again, our sample 'lotEgy' aligned against GenBank accession AM422043 of *N. pubescens*).

Our finding is further corroborated by the molecular phylogenetic results of Smoleń and Falniowski (2010) who found that the other putative Tertiary relict snail species at the locality, *Melanopsis parreyssii*, is, in fact, closely related to a freshwater snail characteristic of central European thermal springs, *Fagotia acicularis* (Férussac, 1823). Whereas their isolation coincides with the onset of the Quaternary, the phylogenetic results clearly separate the subtropical *M. costata* Olivier, 1804 from *M. parreyssii*, and thus hint at morphological convergence between these two species. Moreover, paleontological evidence demonstrate that shell morphology of *M. parreyssii* at Lake Peţea is transforming through geological times and thus can be regarded as an ecological adaptation to warm water (Sümegi et al. 2012a). Additionally, paleoecological reconstructions uncovered the temperate refuge (or oasis) nature of the thermal lake during the last glaciation, not a subtropical one, and the

strata with fossils of *M. parreysii* and *F. acicularis* goes back only to the late Quaternary (Sümegi et al., 2012b).

In light of the above facts, (i) the lack of genetic divergence between *Nymphaea lotus* var. *thermalis* and Egyptian *N. lotus* s.s., (ii) the evident non-Tertiary origin of its habitat, and (iii) the non-Tertiary origin of the co-occurring, presumed relict snail, we conclude the recent occurrence of this species in Lake Peţea, and rule out of its Tertiary relict status. Although our molecular data can suggest the recent occurrence of *N. lotus* in Europe on an evolutionary time scale, the exact timing of introduction is hardly assessable based on our data. According to medieval documents quoted by Negrean (2011), the lake was established as an artificial pond in the 14th century, which makes us suppose the species has a very recent history in Europe. A possible explanation can be, as already mentioned in the literature (Tuzson 1908), the plantation by Turkish during the Ottoman occupation of Hungary, when the Empire extended from North-Africa to central Europe. Ottomans had been known for their advanced horticulture and their devotion to thermal bathes, which makes it likely that *Nymphaea lotus* was planted directly to the thermal lake for ornamental purpose. If we accept this theory, it is still questionable why it was not planted anywhere else in the temperate part of the Ottoman Empire, for example in the hot springs of the Balkan Peninsula and Asia Minor? Another way of introduction could be the epizoochorous and endozoochorous dispersion by migratory birds. However, there is only a few studies that demonstrated the consumption of *Nymphaea* seeds by waterfowls, and there is no exact evidence for seed dispersal of *Nymphaea lotus* by birds at all (Green et al., 2002). We may never be able to find an unequivocal explanation for the presence of *N. lotus* in the thermal spring near Oradea, our data clearly shows it recent introduction to this habitat.

3.1. Conservational remark

Based on four rapidly mutating DNA regions we found no evidence that the only European population of *Nymphaea lotus* var. *thermalis* in Romania is a Tertiary relict. Two more plausible explanations for its origin are plantation of the population in historical times or dispersal mediated by migratory waterbirds. This may imply that we have to regard *N. lotus* var. *thermalis* as a cultural relict in the flora of Europe, and as such, we believe it still has a conservation value similar to as if it was a Tertiary relict. Consequently, we fully agree with the protection of this habitat at the EU level by maintaining the original habitat type ‘Transylvanian hot-spring lotus beds’ as part of the Natura 2000 network.

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5. Declaration of interest

Declarations of interest: none

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Figure captions

Figure 1. Dated phylogeny of a set of species in the genus *Nymphaea* with special focus on subgenus *Lotos* reconstructed using the dataset nrITS and *trn*T-*trn*L together with the plot of speciation rate through time within the lineage. Assessed diversification dates as Mya are placed next by the corresponding node together with 95% HPD bars as grey shadings. Calibration points used for secondary dating are marked with an asterix. Mean rate of speciation is given as a black curve on the graph, the grey area refers to the 95% HPD intervals. Geographic time scale is placed below the tree at the corresponding place.

Figure 2. Haplotype network representing the phylogeographic structure of *Nymphaea lotus* s.l. as reconstructed using the concatenated nrITS and plastid (*trnT*-*trn*L + *psb*M-*trn*D + *rpl*32-*trn*L) sequences. Open circles indicate different haplotypes, whereas black dots refer to hypothetical (unsampled) haplotypes. Size of white circles are proportional to the frequency of haplotypes. Grey dashed line indicates an alternative route in the network. The inserted map shows approximate sampling location for the African samples. *Sample ID abbreviations*: lotNyl: South Africa, Nylsvley Nature Reserve; lotZiw: Ethiopia, Lake Ziwai; lotEgy: Egypt, Nile delta; thePet: Romania, Lake Peţea; zenBuss: Ivory Coast, Grand Bassam