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Genetic Variability of Thermal *Nymphaea* (Nymphaeaceae) Populations Based on ISSR Markers: Implications on Relationships, Hybridization, and Conservation

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Abstract The globally widespread genus *Nymphaea* exhibits a wide range of morphological and taxonomical diversity. The intrusion of a cultivated variety by progressive propagation and its affect on aquatic habitat is demonstrated in this case study. We have studied the genetic diversity, population, and stand structure of the neophyte *Nymphaea*× 'Panama Pacific' as well as other species found in Lake Hévíz and dikes nearby using inter-simple sequence repeat (ISSR) markers. The ISSR assay revealed a low genetic variability for the small populations of *Nymphaea caerulea*, *Nymphaea lotus* var. *thermalis*, and a medium level for *Nymphaea alba*, *Nymphaea rubra* var. *longiflora*, and *Nymphaea*× 'Panama Pacific'. The evolutionary genetic status of individuals found in the overlapping cultivation area of *Nymphaea*× 'Panama Pacific' and *N. caerulea* was affirmed to be of hybrid origin by reticulate network analysis and with morphological parameters. The Bayesian analysis of hybrid classes and the segregation of the ISSR markers also confirmed the hybrid origin of the individuals in question and revealed that they are falling into F2 or latter genotype frequency classes, indicating the viability and fertility of the hybrids. The set of analyzed species by phylogenetic network analysis of ISSR data has been divided into three major groups according to their evolutionary patterns (subg. *Barachyceras*, *Lotos*, and *Nymphaea*). Our results are in accordance with these three major subgenera within *Nymphaea*.

Keywords *Nymphaea* · Inter-simple sequence repeats (ISSR) · Hybridization · Reticulate networks · Genetic diversity

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Introduction

The genus *Nymphaea* L. has a worldwide distribution from the tropics to temperate regions (Verdcourt 1989). The 40–50 species of the genus are phenotypically diverse (Borsch et al. 2007), and this is at least partly due to high level of interspecific polymorphism (Heslop-Harrison 1955) expressed in morphological traits such as leaf size, shape (Volkova and Shipunov 2007), and flower characteristics. The basic haploid chromosome number of the genus is $n=x=14$ (Gupta 1980). The species constitute a polyploid series, with examples of diploids (e.g., *Nymphaea caerulea* Andrews, $2n=2x=28$), tetraploids (e.g., *Nymphaea micrantha* Guill. & Perr., $2n=4x=56$) and hexaploids (e.g., *Nymphaea odorata* Willd., $2n=6x=84$;

Gupta 1978). Hexadecaploids (e.g., *Nymphaea gigantea* Hook, $2n=16x=224$) and aneuploids (e.g., *Nymphaea prolifera* Wiersema, $2n=18$) have also been reported (Langlet and Soderberg 1928). Moreover, cytological intraspecific variation occurs in some taxa, as the somatic chromosome number varies from 42 to 112 in *Nymphaea rubra* Roxb. (Lavania 2002) and in the case of *Nymphaea alba* L. the following number are known: 48, 52, 56, 64, 84, 96, 105, 108, 112, 160 (Gupta 1978; Heslop-Harrison 1955; Bolkhovskikh et al. 1969). The morphological variation in the genus is well studied (Caspary 1879; Conard 1905; Glück 1924; Beug 1961; Radics 1967; Jones and Clarke 1981; Muntendam et al. 1996; Volkova and Shipunov 2007; Szabó 2003) but the genetic diversity of populations, their genetic relatedness, and the status of hybrids have remained less analyzed; only a few studies have addressed these questions (Woods 2003; Woods et al. 2005; Löhne et al. 2008a,b; Werner and Hellwig 2006).

Since the description of the genus *Nymphaea* by Linnaeus (1753), it has been divided into two main infrageneric groups and five subgenera. Caspary (1865, 1888) divided the genus into two sections: *Leptopleura* (with partially fused carpel walls; '*Carpella toto latere libera*') and *Symphytopleura* (mostly complete carpel wall fusion). This was also followed in Conard's (1905) classification, which is still the most widely used system. Conard's (1905) scheme recognizes two majors groups of *Apocarpiae* and *Syncarpiae* reflecting Caspary's sections (Borsch et al. 2007) and five subgenera: *Anecphya* Caspary, *Brachyceras* Caspary, *Hydrocallis* Planchon, *Lotus* DC, and *Nymphaea* DC. Molecular studies of the *rbc*L, *mat*K, and 18 S rDNA or *trn*T–*trn*F regions have been made to study the relationships of Nymphaeales (Les et al. 1999; Qiu et al. 2005a, b; Borsch et al. 2003; Löhne and Borsch 2005). Borsch et al. (2007) utilized the plastid *trn*T–*trn*F region to identify the major lineages of *Nymphaea*. The results of this first molecular study indicated that all subgenera are monophyletic, except for subgenus *Brachyceras*, which appears to be paraphyletic. Löhne et al. (2008a) used nuclear and plastid DNA regions to study the complex reticulate events of subg. *Anecphya*. Borsch et al. (2007) emphasized the necessity of further molecular studies to reveal phylogenetic relationships in all subgenera.

Water lilies can be found in the tropics and as well in the temperate, and even boreal zones because of the broad ecological amplitude (Muntendam et al. 1996). According to Hegi (1965) four species are native in Europe, i.e., *N. alba*, *Nymphaea candida* C. Presl, *Nymphaea tetragona* Georgi, and *Nymphaea lotus* (L.) Willd. The sporadic area of *N. lotus* is uniquely restricted to some hot springs (Masters 1974) that provide exceptional conditions for thermophilic floristic elements. This kind of hot spring can be found for example in Hévíz, Hungary (Fig. 1). It is the largest such spring in Europe (47,500 m^2 surface area) with unique biota due to its chemical composition (reduced sulphuric compounds) and slight radioactivity (caused by radium salts). The natural values of this lake and the surrounding peat land of Quaternary Pleistocene origin were recognized already in the eighteenth century (Bél 1735; Kitaibel 1799). *N. alba* L. (Fig. 2e) has been found in Europe only in Hévíz and in Ukraine (Dubyna 1982). The origin of *N. lotus* var. *thermalis* (DC.) Tuzson (Fig. 2d) is a controversial issue (Szabó 1994). Other *Nymphaea* taxa, such as *N. rubra* var. *longiflora* Lov. (Fig. 2a) and *N. rubra* f. *alba* plus *N. caerulea* (Fig. 2c), were introduced to this site by Sándor Lovassy between 1898 and 1906 (Lovassy 1908). By the early 1980s, a strong interest in the growing of ornamental aquatic plants developed also in Hungary and this led to mass production of several neophytes (Szabó 1994, 2003). The hybrid taxa *Nymphaea*×'Panama Pacific'[1] Tricker (Fig. 2b) was introduced in the lake and later escaped and became naturalized. At least partly due to its fast developing epiphyll vivipary, inherited perhaps from its ancestral taxa, *N. micrantha*, it was able to expand its range very effectively.

Inter-simple sequence repeats (ISSRs) described by Zietkievics et al. (1994) and Kantety et al. (1995) have been used in genomic fingerprinting (Lüdtke et al. 2010; Vargas-Ponce et al. 2011), studies of genetic diversity and phylogenetic analyses (Arif et al. 2009; Gomes et al. 2009; Tanya et al. 2011; Wei et al. 2010), evolutionary biology (Wolfe et al. 1998), and in several other fields. In the genus *Nymphaea* ISSR markers have only been used by Woods et al. (2005) to study the variability and systematics of *N. odorata*. The technique is simple to use providing generally reliable products (Bornet and Branchard 2001), and yield a large number of polymorphism per primer (Ye et al. 2005). The products segregate mostly as dominant (Wei et al. 2010) markers, but with a longer anchoring in the 5′-end some co-dominant bands have also been reported by Fisher et al. (1996) and Provan et al. (1996).

The aims of this study were to (1) investigate the genetic diversity among *Nymphaea* populations from Lake Hévíz and its drainage system, (2) to study phylogeny using phylogenetic network methods, (3) assess genetic relationships among five *Nymphaea* species, and (4) to confirm the status of individuals showing hybrid phenotypic features using ISSR markers.

[1] Later: 'Panama Pacific'

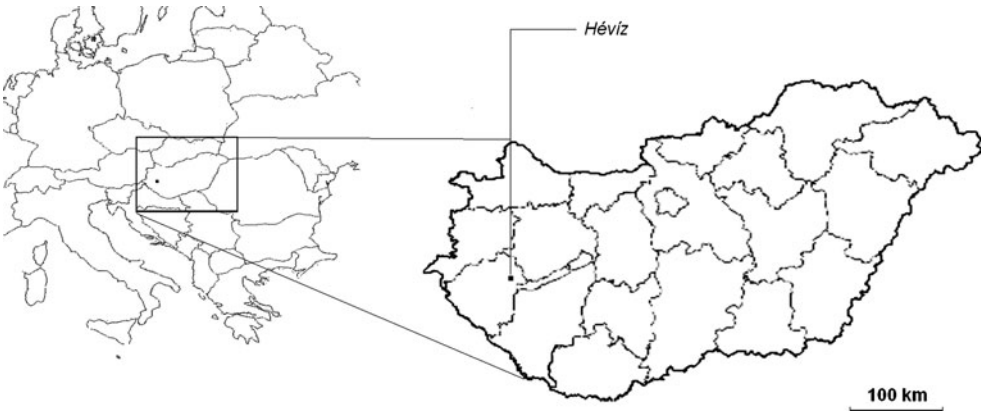

Fig. 1 Geographical location of Lake Hévíz (46.79 ºN; 17.18 ºE) in Central Europe, Hungary

Fig. 2 **a** *N. rubra* var. *long-iflora*, **b** *Nymphaea* 'Panama Pacific', **c** *N. caerulea*, **d** *N. lotus*, **e** *N. alba*, **f** the putative hybrids of *Nymphaea* 'Panama Pacific' × *N. caerulea*

Materials and Methods

Population Sampling and DNA Extraction

Observations on botanical and population dynamics of archaeo- and neophytes have continuously been made for a decade during the biological monitoring program of Lake Hévíz. Samples were collected in August–September 2008 in the lake and along its canalized outlet. Leaf segments were collected randomly from individuals representing different taxa. Leaves of different age were cut and chilled on ice until laboratory treatment or preserved in liquid nitrogen. The number of collected samples is listed in Table 1. DNA was extracted according to the protocol of Kotchoni and Gachomo (2009) with the following modifications: after the samples were suspended in 500 µl extraction buffer 500 µl chloroform/isoamyl-alcohol (24:1) was added and the samples were shaken for 15 min at 250 rpm then centrifuged (8,000 rpm, 10 min). The supernatant was transferred into new Eppendorf tubes and an equal volume of isopropanol was added to precipitate DNA and then centrifuged again (13,000 rpm, 5 min, room temperature). The supernatant was discarded; the pellet was washed with 1 ml of 99% ethanol and 40 µl 3 M sodium acetate, and centrifuged at 13,000 rpm for 5 min at 4°C. The whole washing step was repeated by adding 500 µl of 70% ethanol to the samples. The ethanol was discarded from the pellet by inverting or placing the tubes on a clean paper towel to be air-dried. Finally, the DNA was dissolved in 50 µl TE (Tris-EDTA pH 8.0) and stored at −20°C. The DNA concentration was measured with fluorodensitometric analysis (GeneTools, Syngene, UK) after electrophoresis and ethidium-bromide staining using the GeneRuler 1 kb Ladder (Fermentas, Lithuania) separated on 1% agarose gel; and with a NanoDrop 2000c (Thermo Fisher, USA) spectrophotometer. The DNA quality was also tested directly with performing a test ISSR-polymerase chain reaction (PCR) amplification from all the samples processed in the extractions.

Primer Screening and PCR Optimization

Twenty ISSR primers—with and without anchoring in their 3′ and 5′—were initially screened and optimized on 12 samples for reproducible banding patterns. The optimal annealing temperature and $MgCl_2$ concentration for each primer was determined using gradient PCR (MasterCycler ep384; Eppendorf, Germany) to generate clear banding patterns and optimal PCR conditions. This pilot study was conducted to select primers for further analysis according to their ability to amplify polymorphic products across the samples. Although ISSRs are found to be reproducible (Bornet and Branchard 2001; Ye et al. 2005), because of higher annealing temperature and longer primers (Fang and Roose 1997) replicate experiments were performed containing one negative and positive control, to check the reliability of the primers. Primers generating weak products, or low levels of polymorphism, were excluded from the analysis.

PCR Amplification

After the screening and optimization process, the eight most stabile and reproducible primers were selected to detect genetic variability among 48 samples (Table 2). Amplification reactions were performed in 10 µl volumes containing 5 µl nuclease-free water, 20 ng template DNA, 0.5 µM primer, 0.2 mM dNTP (Fermentas, Lithuania), 1 µl 10× PCR buffer (1 mM Tris-HCl, pH 8.8 at 25°C, 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100), and 0.5 U of DynaZyme II (Finnzymes, Finland) polymerase. All reactions were done in a MasterCycler ep384 (Eppendorf, Germany) with the following conditions: 2 min at 94°C for

Table 1 List of species with collection locality used in the analysis

Taxon name	Subgenus	Section	Locality	Pop. Code	Pop. Size	*N*
N. alba	*Nymphaea*	*Nymphaea*	Effluent	Namt001-5	ca. 40	5
N. caerulea	*Brachyceras*	–	Lake	Nct001-2	2	2
			Effluent	Nck001-5	ca. 50	5
N. lotus var. *thermalis*	*Lotos*	–	Effluent	Ntk001-3	ca. 40	3
Nymphaea cv. 'Panama Pacific'	*Brachyceras*	–	Lake	Nmppt001-7	ca. 120	7
			Effluent	Nmppk001-7	ca. 200	7
N. rubra var. *longiflora*	*Lotos*	–	Lake	Nrt001-10	ca. 180	10
			Effluent	Nrk001-2	ca. 10	2
N. rubra var. *longiflora* f. *alba*	*Lotos*	–	Lake	Nrtf001-4	ca. 25	4

Pop. Code the abbreviation used in the phylogenetic network analysis, *Pop. Size* approximate number of individuals in the lake, *N* number of random samples collected. Putative hybrid individuals of *N. caerulea*×Panama Pacific are listed as Nck0?6 and Nck0?7

initial denaturation, 35 cycles of 30 s denaturation at 94°C, 1 min annealing at the primer specific temperature (Table 2), and 2 min extension at 72°C, followed by a final extension for 5 min at 72°C. Amplification products were separated on 1.5% agarose gels (Promega, USA) in 0.5× TBE buffer (300 V, 1.5 h) and post-stained with ethidium bromide. The gels were documented using the GeneGenius Bio Imaging System (Syngene, UK).

Band Scoring and Data Analysis

The evaluation of the banding patterns was carried out with the program GeneTools (Syngene, UK). Only well-resolved, distinct bands were scored; where reliable bands are considered as amplicons found in replicate reactions. The amplified fragments were coded in an absence/presence (0/1) data matrix. It was presumed that fragments with equal length were amplified from corresponding loci and represent a single, dominant locus with two possible alleles. To measure the information content detected with each primer the polymorphic information content (PIC) value was calculated according to the formula of (Botstein et al. 1980), while the heterozygosity (H) value was calculated according to Liu (1998). As PIC and H are both influenced by the number and frequency of alleles, the maximum number for a dominant ISSR marker is 0.5, since two alleles per locus are assumed in the analysis (Henry 1997; De Riek et al. 2001; Bolaric et al. 2005).

To characterize the genetic diversity in the *Nymphaea* populations the percentage of polymorphic bands; expected heterozygosity or Nei's gene diversity (H_E; Nei 1973); Shannon's index of phenotypic diversity (I; Lewontin 1972), and the coefficient of genetic differentiation (G_{st}) was calculated using POPGENE v.1.32 (Yeh et al. 1999). Where, the H_E was calculated from allele frequencies based on the square root of the frequency of the recessive allele. Gene flow (N_m) was estimated from G_{st} values (McDermott and McDonald 1993) to measure the genetic interaction among populations found in the lake and in the outlet. To further assess population structure, a principal coordinate analysis (PCA) was performed based on Nei's genetic diversity index matrices (Nei and Li 1979). This analysis was carried out with GenAlex v. 6.0 (Peakall and Smouse 2006).

Neighbor-net Analysis

We carried out a network analysis to study the genetic relationships of the populations and individuals, as well as to reconstruct the phylogenetic relationships and origin of the taxa examined. A neighbor-net (NNet) analysis was carried out with SPLITSTREE v. 4.6 (available at http://www.splitstree.org/) because evolutionary models based on split networks add extra topology-related parameters to phylogenetic analysis, allowing the constructed network to fit the data better than individual trees (Huson and Bryant 2006). The distribution of incompatible splits based on uncorrected p distances were inferred and represented as a splits graph, because we wanted to visualize conflicting signals in the data sets, whether they arise from sampling error or genuine recombination (Bryant and Moulton 2004). With the possibility to represent alternative phylogenetic histories in the analysis (recombination, hybridization, gene transfer), we desired to extract phylogenetic signal not detected with tree-based methods (Esser et al. 2004; Fitch 1997). One sample from *Nuphar lutea* (L.) Sm. was used as an outgroup. A recombination network was also constructed from the samples of *N. rubra* var. *longiflora* and *N. caerulea* including their putative hybrids with common phenotypic characters to visualize the reticulating patterns between the three groups.

Table 2 Details of the primers used in the analysis

Primers	Sequence 5′–3′	Annealing temperature	Total amplified products	H value	PIC value
UPG-01	$GAG(CAA)_5$	55	21	0.26	0.22
UBC-811	$(GAGA)_4C$	48	24	0.37	0.31
UBC-845	$(CT)_8AGG$	50	19	0.25	0.21
UBC-852	$(TC)_8AGA$	53	36	0.22	0.21
UBC-868	$(GAA)_6$	45	21	0.21	0.19
OP3	$(GACA)_4$	45	24	0.38	0.33
ISSR-9	$(AC)_8YA$	53	21	0.29	0.26
ISSR-19	$(GAT)_4TGG$	53	33	0.30	0.26
Mean	–	–	24.87	0.29	0.25
Total	–	–	199	–	–

Hybrid Class Identification

We carried out a model-based Markov chain Monte Carlo simulation in a Bayesian analysis based on the method of Anderson and Thompson (2002) to compute the posterior probabilities of possible genotype classes. We pre-defined four hybrid classes as F1, F2 and backcross to 'Panama Pacific' or to *N. caerulea*. The scored 199 ISSR loci were transformed into 'lumped data format' accepted by the program NewHybrids PC 1.1 (Anderson and Thompson 2002) where the markers were coded as '+'present or '–'absent. The calculations were carried out using all collected individuals from *N. caerulea* and 'Panama Pacific'. Jeffrey's prior π and θ parameters were used to calculate the posterior probabilities, which were based on 1,000,000 iterations, following a burn-in period of 100,000.

Results

We analyzed the historical distinctiveness of the naturalized and indigenous populations by utilizing the appropriate ISSR markers. This molecular marker assay enabled us to estimate the degree of genetic linkage among the different *Nymphaea* populations via gene flow and to detect genetic variability. In the ISSR analysis, a total of 199 bands were scored, of which 175 (87.95%) were polymorphic. In average, we detected approximately 24 bands per primer in each reaction. The band size ranged from 150 to 3,000 bp. The primer UBC-852 amplified the largest number (36) of products while UBC-845 created only 19. The average PIC value was 0.24 while the average *H* value was 0.29, as calculated for the applied primers. Considering that the highest value for dominant markers is 0.5, the average degree of polymorphism detected with the primers is moderate. The primer OP3 containing four tandem repeats of GACA revealed the highest value (0.33) for both PIC and *H* (0.38) and produced the largest number of markers discriminating among and within the analyzed individuals. The UBC-868 primer generated the lowest value for both PIC (0.19) and *H* (0.21). Further details about the primers and PIC or *H* values are summarized in Table 2.

In total, there were 72 polymorphic bands (36.18 %) detected in *N. rubra* var. *longiflora* population. The *N. lotus* var. *thermalis* population had the lowest (32; 16.08%), and the 'Panama Pacific' population the highest number of polymorphic bands and genetic variability (90; 45.23%). We have detected a total of 47 (23.62%) polymorphic bands in the *N. caerulea* and 46 (23.12%) in *N. alba* populations (Table 3). Two putative hybrid individuals were identified. Between these hybrids, nine (4.52%) polymorphic bands were detected. Levels of ISSR variation within populations varied among the populations (Table 2). The average H_E ranged from 0.0187 to 0.1140. Across all populations, the mean expected heterozygosity was 0.1776. In the populations of *N. rubra* var. *longiflora* (I=0.1734, H_E=0.1140), 'Panama Pacific' (I=0.1683, H_E=0.1059), *N. alba* (I=0.1506, H_E=0.1067), and in the white flowered *N. rubra* var. *longiflora* f. *alba* (I=0.1345, H_E=0.1034) the genetic diversity was higher than in the *N. caerulea* (I=0.1082, H_E=0.0699) and *N. lotus* var. *thermalis* (I=0.0859, H_E=0.0567) populations. Compared to the small size of the overall population, we detected low value of genetic differentiation and relatively high value of gene flow within *N. rubra* var. *longiflora* (G_{st}=0.3945, N_m=0.7569), *N. caerulea* (G_{st}=0.3301, N_m=1.0489), and 'Panama Pacific' (G_{st}=0.198, N_m=2.0592) populations.

The split graph (Fig. 3), based on uncorrected *p* distance matrixes inferred with the NNet method clearly separate the analyzed samples into three distinctive groups. The first, which contains the samples of *N. rubra* var. *longiflora* collected from the lake and the outlet populations coupled with samples from the small *N. lotus* var. *thermalis* population. In the *N. rubra* group, a small but characteristic subgroup of the subpopulation of white flowered *N. rubra* var. *longiflora* f. *alba* individuals is evident. The samples

Table 3 ISSR analysis of genetic diversity in *Nymphaea* populations at Lake Héviz and the connecting outlet

Population	NPB	PPB%	*I*	H_E
N. rubra var. *longiflora*	72	36.18	0.1734	0.1140
N. rubra var. *longiflora* (white)	47	23.62	0.1345	0.1034
N. lotus	32	16.08	0.0859	0.0567
N. caerulea	47	23.62	0.1082	0.0699
Putative hybrids	9	4.52	0.0273	0.0187
Nymphaea 'Panama Pacific'	90	45.23	0.1683	0.1059
N. alba	46	23.12	0.1506	0.1067
Average	49	24.63	0.1212	0.0822
Across all populations	175	87.95	0.2943	0.1776

NPB number of polymorphic bands, *PPB* percentage of polymorphic bands, *I* Shannon's index of diversity, H_E expected heterozygosity

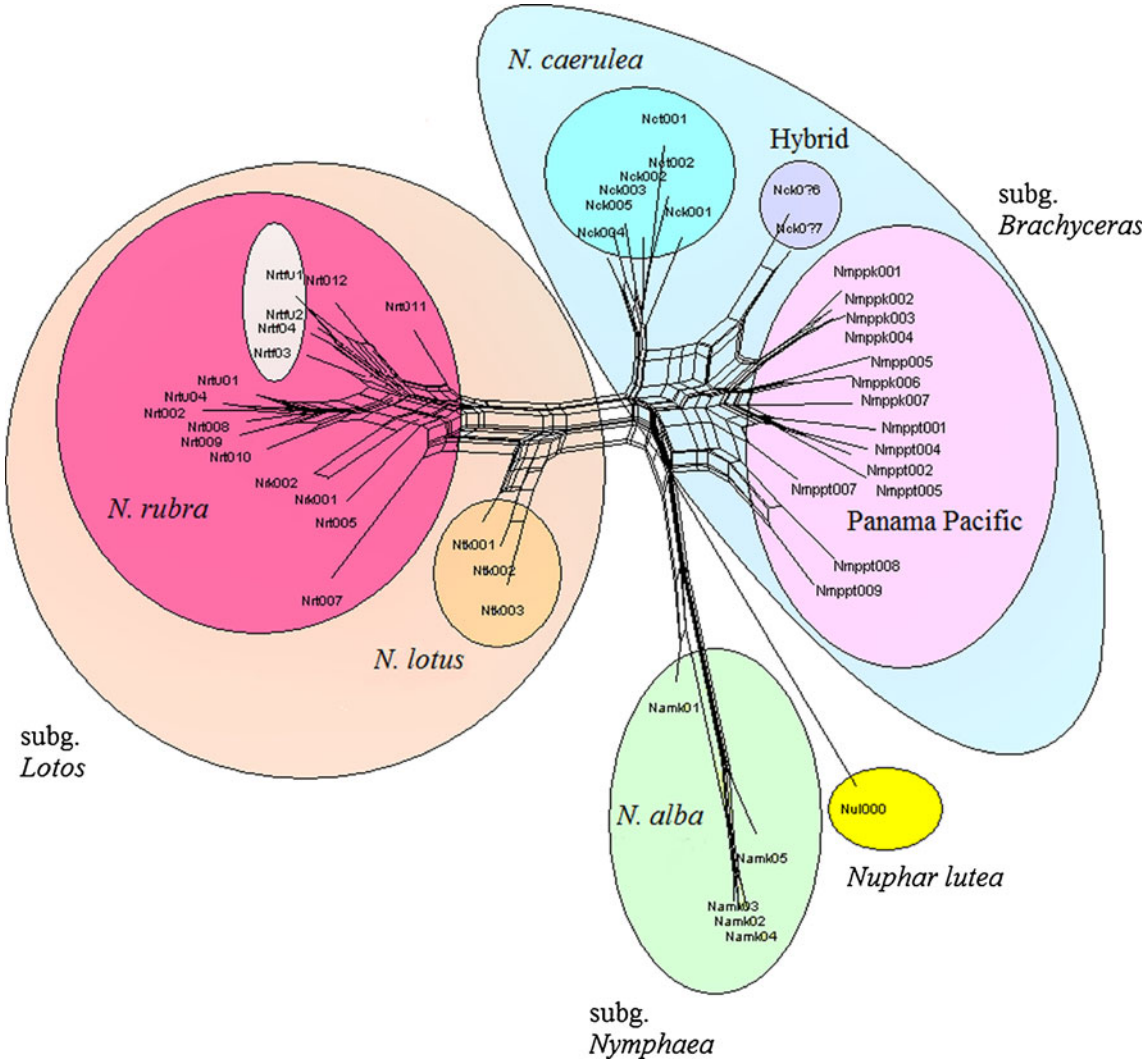

Fig. 3 Distribution of incompatible splits calculated from the ISSR data. The split graph is based on the NNet method calculated from the uncorrected *p* distances. The overall topology shows the relationships of *Nymphaea* species and specimens examined in the study

collected in the lake from the *N. rubra* populations grouped together and split from the two samples which were collected in the outlet. Laterally, linking graph lines (parallel edges) are found between the outlet and lake populations suggesting gene flow. Interestingly, three samples collected in the lake are outside of these main groups. Clear lateral lines represented obvious genetic relationships among the samples of *N. lotus* var. *thermalis*. The second major group is formed by the samples of the indigenous *N. alba*. This small population can now only be found in the outlet. The third major group was composed by *N. caerulea* and 'Panama Pacific' where four small subgroups can be distinguished.

Putative hybrids were resolved in a separate group as intermediate between *N. caerulea* and 'Panama Pacific' with parallel edges. The same intermediate position is shown in the PCA plot (Fig. 4). The separately performed recombination network analysis emphasizes parallel edges between 'Panama Pacific', *N. caerulea*, and hybrid individuals (Fig. 3). The Bayesian analysis of hybrid classes resulted in posterior probabilities ranging from 0.962 to 0.998 for the proposed parental genotypes (*N. caerulea* or 'Panama Pacific'; Fig. 5). Both hybrid individuals had posterior probabilities of P=0.8956 to be regarded as F2 hybrids, and probabilities of P=0.0014 and P=0.1023 to fall in F1 class, and/or a backcross to 'Panama Pacific' (Fig. 6).

Discussion

Genetic Diversity and Population Structure

Information about genetic diversity can provide insights into the demographic history and origin of the analyzed population

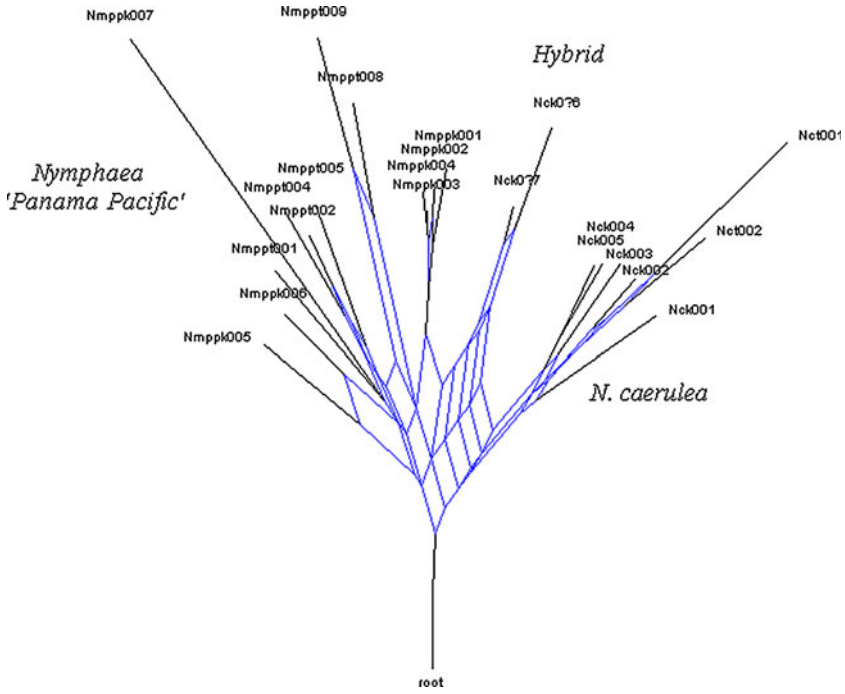

Fig. 4 Recombination network constructed from the ISSR binary sequence data to represent hybridization events between the putative hybrids and their two ancestral taxa. The recombination nodes are annotated and labeled with color *blue* to describe which parts of the two putative ancestors binary sequences are part of the hybridization event

or taxa (Milligan et al. 1994). In addition to understanding the importance of processes creating diversity within and among populations (gene flow, genetic drift, and selection), it is important to assess future risk of erosion of diversity, and to plan effective conservation strategies based on the data of genetic diversity (Neel and Ellstrand 2003). We observed low genetic diversity among and within the populations of *Nymphaea* in Lake Hévíz and in adjoining ditches. Relatively small number of genetic studies has been published on naturalized and/or indigenous *Nymphaea* populations. Woods et al. (2005) found high interpopulation variability in *N. odorata* using ISSR markers. Results correspond to others obtained by different marker systems in diverse outcrossing organisms. The observed low genetic variability could result from the self-pollinating breeding system of *Nymphaea* populations; however, this system is poorly understood (Endress 2001). According to Bernhardt et al. (2003), some species are self-compatible (e.g., *N. capensis*

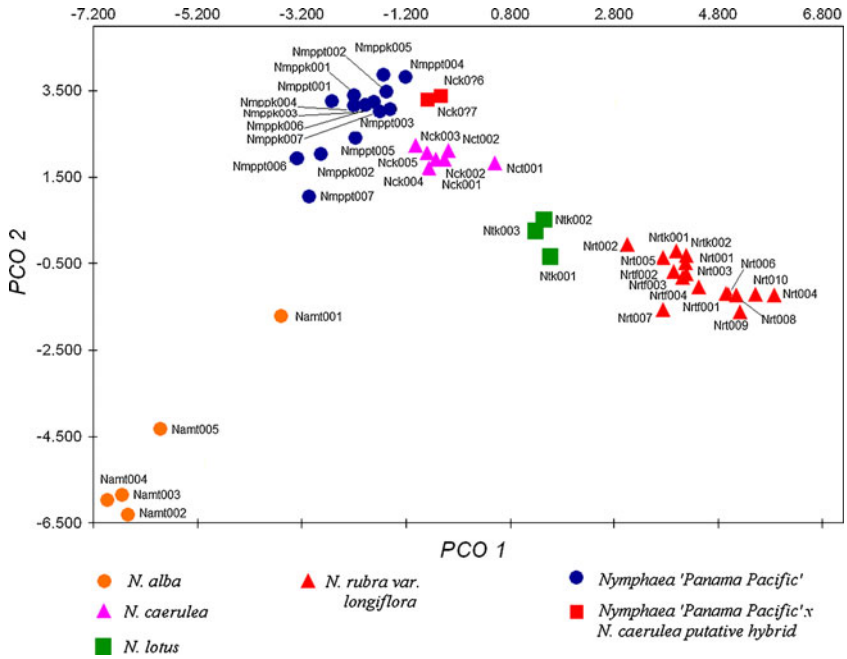

Fig. 5 Principle coordinates analysis based on Nei and Li distances using 199 ISSR markers from *Nymphaea* population. Abbreviations in Table 1

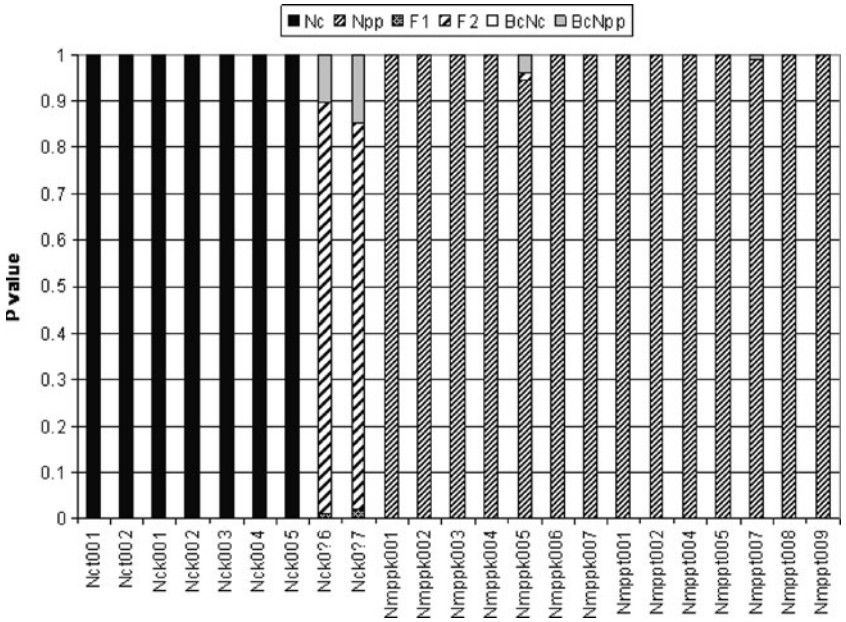

Fig. 6 Posterior probabilities of genotype frequency classes inferred from the ISSR data of the putative hybrids and their ancestral species. *Nc*, *Nymphaea caerulea*; *Npp*, *Nymphaea* 'Panama Pacific'; *F1*, F2 generation; *BcNc*, backcross to *N. caerulea*; *BcNpp*, backcross to *Nymphaea* 'Panama Pacific'

Thunb., Orban and Bouharmont 1995; *N. lotus*, Hirthe and Porembski 2003) while others are bisexual and protogynous (e.g., *N. alba*, Heslop-Harrison 1955), or homogamous (e.g., *Nymphaea jamesoniana* Planch., *Nymphaea lingulata* Wiersema, and *Nymphaea ampla* DC., Wiersema 1988). It has been assumed that the outermost anthers open already on the first day of anthesis and do not open when the stigma becomes receptive (Endress 2001). However, self-pollination is only a hypothesis, which has not been proven by our analysis, but can be clarified by further pollination experiments.

Another possible explanation for the observed low genetic variability could be the mating of genetically related and geographically closely distributed individuals as previously presented by Zong et al. (2008). In the case of *N. rubra* var. *longiflora* (rhizome proliferation) and 'Panama Pacific' (leaf vivipary), the vegetative reproduction could be a reasonable explanation for the low genetic diversity. However, this does not apply to *N. caerulea* and *N. lotus* var. *thermalis*. What can be the cause of low genetic diversity detected for these two taxa? It could be expected that the number of ISSR bands would be affected by ploidy level influencing distance based NNet algorithms and modifying overall topology as well as genetic diversity indices. This explanation seems unlikely, since no significant difference was recorded in the number of bands within and among ploidy levels. However, polyploidy still can be a reasonable implication of lower genetic diversity detected for these two diploid species. Soltis and Soltis (2000) previously concluded that polyploid populations generally maintain higher levels of heterozygosity than diploids and exhibit less inbreeding depression. Another alternative hypothesis might be related to the historical occurrence of *N. lotus* var. *thermalis* in the lake, which is considered as an indigenous pre-glacial relict in the Carpathian Basin. The occurrence of *N. lotus* var. *thermalis* in Lake Hévíz is a matter of controversy that has been discussed in several historical studies (Szabó 1994, 2003). Based on early descriptions, the indigenous status of this white-flowered water lily has not been documented unambiguously. During the nineteenth century, *N. lotus* appeared and disappeared in the like (Szenczy et al. 1842), e.g., it was present between 1826 and 1842 (Szabó 1994, 1997). Lovassy received rhizomes from Nagyvárad (Oradea) that originated from Budapest University Botanical Garden in 1898. He reported the lack of ability of vegetative reproduction (Lovassy 1908). Szabó brought four rhizome segments from Pece near Nagyvárad and made experimental pots in the temperate waters of the lake's outlet in 1997 (personal communication). It seems safe to assume that the current population in the canalized outlet could have originated from sources with low genetic diversity regardless of repeated introductions. Nowadays, a small number of individuals grow in the outlets which are endangered by the gradation and fast spread of 'Panama Pacific' and several other macrophytes. Low genetic diversity is demonstrated also by *N. caerulea* or by relic *N. alba* population at the lower stretch of the outlet, which has not yet been invaded by 'Panama Pacific'. There is clear gene flow among the populations, decreasing the G_{st} and increasing the N_m values. This connection could be attributed to the intensive water flow from the lake to the outlet.

Implications on Genetic Relationships

The phylogeny of the genus *Nymphaea* has been intensively studied since the first molecular studies were published.

However, there are still open questions and new data has also been generated that is now available for reconstruction of robust hypothesis of phylogeny (e.g., Volkova et al. 2010). Outgroup effects in phylogenetic analyses of the genus have been thoroughly discussed in the first molecular studies, as well as the identification of major lineages and biogeographic patterns (Slocum 2005; Borsch et al. 2007; Löhne et al. 2008b). In the present analysis, we used *Nuphar lutea* as outgroup, while it was concluded that distant or closely related outgroups will not affect the basic topology of reconstructed trees (Borsch et al. 2008). In this analysis, the relationships of five taxa belonging to three different subgenera were studied and relationships among them represented by a neighbor net. Each taxon was clearly separated according to their taxonomic position by the split graph constructed from the ISSR data. Three major groups can be outlined: subg. *Brachyceras*, subg. *Lotus* and subg. *Nymphaea* (Fig. 2). The results of the present study using multi locus inter-sample sequence repeat markers were in accordance with Borsch et al. (2007) based on chloroplast *trn*T–*trn*F sequence data. The present work is the first one to study genetic relationships of *Nymphaea* using neighbor-net construction. This method could be used in the future to demonstrate incongruence found in the data set or even to study recent microevolutionary processes which involve reticulating events (e.g., hybridization). The highly variable chromosome numbers within *Nymphaea* suggest that polyploidy and reticulate evolutionary events can be the driving forces of speciation. Löhne et al. (2008a) found complex reticulating patterns in Australian water lilies (subg. *Anecphya*) based on the analysis of nuclear and plastid DNA sequences. Further analyses should be aimed to reveal whether reticulate evolution acts in the whole genus or if it is just a special feature of separate infrageneric groups.

Status of the Putative Hybrids

The area where two distinct species meet to interbreed and hybridize is the fertile ground for evolutionary studies concerning models of speciation, selection, and recombination (Anderson and Thompson 2002). In conservation biology, hybridization between indigenous and naturalized species or between wild and cultivated ones is a topic of great concern. Two individuals showing hybrid morphological patterns between *N. caerulea* and 'Panama Pacific' were found in the channel system. Besides the hybrid morphological patterns, they were located in the overlapping zone of the populations of *N. caerulea* and 'Panama Pacific'. Hybrids between different taxa are often found in the overlapping zones of species, called hybrid zones (e.g., *Quercus*, González-Rodríguez et al. 2004). The first evidence in such cases can be the morphological traits inherited from both parents. Intergradations between two differentiated populations or taxa is represented by morphological and genetic intermediacy (Beckstrom-Sternberg et al. 1991; Durett et al. 2000), molecular markers can provide a large number of neutral and independent characters that are extremely useful in the genetic analysis of hybrids (Rieseberg and Ellstrand 1993). The putative hybrids in our present study possess epiphyll vivipary and have dentate leaf margins like 'Panama Pacific'; while the sepals are lanceolate and on the abaxial side of the leaf blades, purple spots can be found that are typical for *N.*

Table 4 Character states of seven diagnostic morphological markers assayed in the putative *Nymphaea* 'Panama Pacific' × *N. caerulea* hybrids

Morphological trait	*Nymphaea* 'Panama Pacific'	Hybrid	*N. caerulea*
Epiphyll vivipary	+	+	−
Dentate or serrated leaf edge	+	+	−
Small purple spots			
On the abaxial of the leaf	−	+	+
On the sepals	−	+	+
Flower color			
Light violet blue	−	−	+
Dark purple	+	−	−
Light violet–purplish (intermediate)	−	0	−
Sepals			
Lanceolate	−	+	+
Ovate	+	−	−
Petals			
Lanceolate	−	−	+
Ovate	+	−	−
Midly-lanceolate (intermediate)	−	0	−

Character states typical of *Nymphaea* 'Panama Pacific' and *N. caerulea* are denoted with '+' or '−'. Intermediate states are denoted as '0'

caerulea. The flower color is intermediate between dark purple and light violet of the parents. Further morphological traits are summarized in Table 4. In the molecular analysis, we have detected additive banding patterns between the populations of 'Panama Pacific' and *N. caerulea*. Some ISSR markers were present in 'Panama Pacific' and in the hybrids, but they were absent or present in *N. caerulea* and in the hybrids and absent in 'Panama Pacific'. The presence of diagnostic ISSR markers and morphological markers confirm the hybrid origin of the water lilies found in the overlapping zone. Despite well-known disadvantages (e.g., comigration of bands that are paralogous; heteroduplex formation, or collision of bands), ISSR markers are suitable for the detection of hybridization and introgression. The recombination network constructed from the ISSR binary data also confirms the hybrid status of the individuals in question, as well as the results of the principle coordinate analysis (Fig. 4). In the PCA plot, the hybrids are in an intermediate position between their possible ancestors. The separation of the hybrids confirms the findings of Young et al. (2001) where PCA was also used successfully to separate hybrid individuals from their parents.

Recombination networks are promising representations for evolutionary events like hybridization, allopolyploidization, or diploid hybrid speciation because they describe the above processes as a net due to gene transfer from both ancestral species. Split networks must be viewed in a more abstract way as they give a visual representation of incompatible signals in certain parts of phylogeny (Huson and Steel 2004). They also generate high portion of false negative results and for this reason the results should be handled with substantial caution (Bell and Hyvönen 2010; Poczai and Hyvönen 2011). Contrary to split NNets, recombination networks can be interpreted directly. However, recent studies have revealed that there are strong connections between split networks and reticulate networks (Huson et al. 2005; Huson and Kloepper 2005). This case study of *Nymphaea* hybrids supports that both the split network and recombination network signals represent and detect the same hybridization event between the two ancestral species and their hybrids. The Bayesian analysis of posterior probabilities successfully elucidates that the hybrids belong to a later generation, possibly F2. This result suggests that the hybrids are viable and fertile. It can be concluded that introgression, also in closely related *Nymphaea* species, could be a source of increased genetic variability.

Implications for Conservation Management

Accurate estimates of genetic diversity are important for conserving and preserving rare plants (Zhang et al. 2005). Knowledge of the levels and distribution of genetic diversity is important for planning conservation strategies for protected populations (Qiu et al. 2005a, b). Therefore, data from different molecular markers provide valuable information to identify the appropriate units of biodiversity for conservation purposes (Chen et al. 2010). All populations we studied have low degree of genetic diversity, which should rapidly decrease if the population size reduces because of the loss of habitat. The hybrid neophyte 'Panama Pacific' appears to displace the other species both in the lake and in the connecting ditches. The fragmentation of the populations can cause bottlenecks which result in the loss of rare alleles occurring in low frequency and this will affect the genetic variability due to the effects of genetic drift (Allendorf and Luikart 2007; Takaki et al. 2009). During the biomonitoring process, we observed the great abundance of 'Panama Pacific' in the lake but only a few individuals can be found in the outlet. This species possess epiphyll vivipary. This effective strategy makes it possible to occupy large area in a very short time. Bearing young plants at the junction of leaf blade to the petiole—which is inherited from its parent *N. micrantha*—makes this species a successful and aggressive competitor as compared to other species found in the lake. Young plantlets may bloom while still attached to the mother plant. This vegetative invasion undoubtedly contributed to the decrease of genetic variability in the population. An important task would be the optimal management of the aggressive 'Panama Pacific' which endangers the habitat of the other important species. This aggressive dispersion strategy is encouraged by the inappropriate dredging of the water course. Because of this, the 'Panama Pacific' population gains a remarkable advantage over other species without epiphyll vivipary. The solution would be the selective mechanical control of the neophyte water lilies in the lake and the outlet.

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