

Reinterpretation of *Potamogeton ×nerviger*: solving a taxonomic puzzle after two centuries

Identyta *Potamogeton ×nerviger* rozluštěna dvě století od jeho objevu

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Hybrids form an important component of *Potamogeton* diversity but their exact taxonomic identities and distributions are often insufficiently known. *Potamogeton nerviger* was described from Lithuania in 1827 as a proper species. Based on morphological and anatomical characters, its interpretation has since varied, ranging from synonymization with other species to identification as different hybrids and intraspecific taxa. Currently, it is universally recognized as the hybrid *P. alpinus* × *P. lucens*. Using a combined molecular, morphological and anatomical investigation we re-examined the identity of *P. ×nerviger*, based on both original and recent plant material. We report a successful amplification and sequencing of nuclear ribosomal ITS1 region from a 188-year-old type collection. This was shown to be genetically identical to the morphologically matching plants recently collected at the type locality. Comparison with molecular characters of the possible parental species shows that *P. ×nerviger* is not *P. alpinus* × *P. lucens*, as currently believed, but another hybrid, *P. nodosus* × *P. perfoliatus*, which is currently called *P. ×assidens*. This molecular identification is also supported by anatomical evidence. In contrast, the actual existence of the hybrid *P. alpinus* × *P. lucens* is doubtful. Consequences for nomenclature and identities of records reported from other sites are discussed.

Key words: herbarium, hybrid identification, hybridization, internal transcribed spacer, molecular identification, taxonomy, *Potamogeton*, type specimen

Introduction

Potamogeton is a genus of aquatic plants whose taxonomy is complicated by the occurrence of numerous hybrids (Preston 1995, Wiegleb & Kaplan 1998, Zalewska-Gałosz 2002, Kaplan 2010). Even though the first *Potamogeton* hybrids were recorded more than a century ago (reviewed by Kaplan et al. 2009) and later detected in many localities and countries, still little is known about their total diversity and distribution (Zalewska-Gałosz & Ronikier 2010, 2012, Kaplan et al. 2011). Previous studies have demonstrated that due to extensive phenotypic plasticity, some *Potamogeton* hybrids can mimic species, and vice versa (Kaplan 2002, 2005a, Kaplan & Fehrer 2004, 2009, Kaplan & Wolff 2004, Kaplan & Symoens 2005, Kaplan et al. 2009). Although hybrid origin is usually detected based on morphological characters of the given hybrid individual, straightforward identification of

both parents is sometimes very difficult (e.g. Preston 1995, Zalewska-Gałosz 2010, 2011). Consequently, many local forms are poorly known and their exact taxonomic identities are unclear (e.g. Kaplan 2005b, Kaplan & Symoens 2005, Kaplan & Marhold 2012). Since morphological identification can sometimes be misleading even for experts, the exact identity of some hybrids can be revealed only by molecular analysis (Kaplan et al. 2009, Zalewska-Gałosz et al. 2010, Kaplan & Fehrer 2011, Zalewska-Gałosz & Ronikier 2012). In recent years, application of DNA-based molecular methods has contributed substantially to *Potamogeton* taxonomy, especially in further unravelling the complexity of hybridization processes in the genus. Molecular data has provided decisive support for identification of several new hybrid combinations (Kaplan et al. 2009, 2011, Zalewska-Gałosz et al. 2010, Kaplan & Fehrer 2011, Zalewska-Gałosz & Ronikier 2011, Bobrov et al. 2013) and for establishing the actual origin of previously described, erroneously interpreted hybrids (Kaplan & Fehrer 2011). Furthermore, analyses of ITS sequences enabled recognition of the existence of a triple hybrid (Kaplan & Fehrer 2007).

Potamogeton nerviger was described as a proper species by J. F. Wolfgang almost two centuries ago (Schultes & Schultes 1827). Duplicates of the original collection were widely distributed (Kaplan & Zalewska-Gałosz 2004) and studied by several *Potamogeton* experts, who interpreted its identity in various ways. Earlier authors had regarded *P. nerviger* as conspecific with *P. alpinus* (Bennett 1889), sometimes recognizing it as infraspecific taxon *P. alpinus* var. *purpurascens* subvar. *nerviger* (Ascherson & Graebner 1897, Graebner 1907). Fischer (1907) suggested that it might be a hybrid between *P. alpinus* and *P. lucens*. Hagström, a monographer of *Potamogeton*, carefully examined the original plants and considered them to be identical with the British hybrid *P. ×griffithii* (Hagström 1916), which is *P. alpinus* × *P. praelongus* (Dandy & Taylor 1939, Preston 1995). Interestingly, soon after his monograph was published, Hagström changed his mind and in 1919 annotated the original herbarium specimen of *P. nerviger* kept in his private collection (now preserved at LD) as *P. nodosus* × *P. perfoliatus*. This view, however, has never been published. Dandy (1958, 1975) and Dandy & Taylor (1967) followed Fischer's view on the identity of *P. nerviger* and regarded it as a hybrid between *P. alpinus* and *P. lucens*. This identity was adopted in later taxonomic publications and currently is widely accepted (e.g. Stace 1991, Czerepanov 1995, Preston 1995, 2015, Wiegleb & Kaplan 1998, Trei et al. 2003, Kaplan & Zalewska-Gałosz 2004, Wiegleb et al. 2008).

In 2009, during studies on the hybrid between *P. perfoliatus* and *P. nodosus*, which was finally described as *P. ×assidens* (Zalewska-Gałosz et al. 2010), we noticed its morphological similarity to *P. ×nerviger*. Both entities shared some diagnostic characters, such as semiamplexicaul submerged leaves, which pointed at *P. perfoliatus* or *P. praelongus* as one of the parents, as well as well-developed lacunae along the midrib and the lateral veins, a character which could have been inherited from *P. nodosus*. Both hybrids differed, however, in stem anatomy. For *P. ×nerviger*, Hagström (1916) reported the occurrence of cortical strands in the cortex. In contrast, none of the anatomical samples of *P. ×assidens* that we examined contained any cortical strands, which is consistent with the stem anatomy of its parental species (Zalewska-Gałosz et al. 2010). Because stem anatomy has high diagnostic value in *Potamogeton* and has been successfully used for identification of species and hybrids with different anatomic patterns in a number of studies (e.g. Raunkiaer 1896, 1903, Fischer 1904, 1905, 1907, Hagström 1916, Ogden 1943,

Symoens et al. 1979, Wiegleb 1990a, b, Kaplan 2001, 2005a, b, Kaplan & Symoens 2004, 2005, Kaplan & Wolff 2004, Zalewska-Gałosz et al. 2009, Zalewska-Gałosz 2010, 2011), and because no fresh plants of *P. ×nerviger* were available for comparison and molecular analyses at that time, we tentatively considered these two hybrids as different.

In 2008 we collected from the Verknė river, the type locality of *P. ×nerviger*, individuals morphologically corresponding to this hybrid. Molecular analysis presented in this work proved that these individuals are in fact hybrids between *P. nodosus* and *P. perfoliatus*, the hybrid taxon described as *P. ×assidens* (Zalewska-Gałosz et al. 2010). The morphological similarity of both hybrids and potential co-occurrence of two rare *Potamogeton* hybrids in the same river inspired us to conduct a molecular and anatomical study of the type specimen of *P. ×nerviger* deposited in KRA.

The aims of this contribution are: (i) to test, using molecular methods, whether the type collection of *P. ×nerviger* really represents a hybrid between *P. alpinus* × *P. lucens*, as recognized recently by all authors; (ii) to provide a detailed survey of morphological and anatomical characters of *P. ×nerviger*, and finally, if the previously suggested identity is excluded; (iii) to unequivocally identify the actual parentage of this mysterious hybrid.

Materials and methods

Plant material used for molecular analysis

DNA was extracted from a half of the leaf taken from the syntype of *Potamogeton ×nerviger* deposited in KRA and collected prior to 1827 (Kaplan & Zalewska-Gałosz 2004). Additionally, two individuals from a rich and uniform colony of plants morphologically matching *P. ×nerviger* were sampled at its type locality, namely the Verknė river 1 km south of Lielionys, at the village of Stakliškės, Lithuania (55°33'26.0"N, 24°20'36.8"E; Fig. 1) and cultivated in the Experimental garden of the Institute of Botany, Průhonice, Czech Republic (Fig. 2). An aliquot of the DNA extracted from the type of *P. ×nerviger* was deposited at the Institute of Botany, Jagiellonian University. As a reference for the analysis of the putative hybrid genotype, a comprehensive set of typical broad-leaved *Potamogeton* species representing potential parental taxa was included in the molecular analysis (Table 1, Table 2). The origin of these samples is provided in Zalewska-Gałosz et al. (2009, 2010) and Zalewska-Gałosz & Ronikier (2012). Voucher specimens from the field were deposited in the Herbarium of the Institute of Botany, Jagiellonian University (acronym KRA), those from the cultivation are preserved in the herbarium of the Institute of Botany, The Czech Academy of Sciences, Průhonice (acronym PRA).

All species and hybrids included in this study are tetraploids, with the exception of *P. polygonifolius*, which is diploid (Kaplan et al. 2013). The previous studies showed that ITS sequences of *Potamogeton* species are well-homogenized even in polyploids, indicating diploidization of the genome (Kaplan & Fehrer 2007, Kaplan et al. 2013), therefore making them suitable for hybrid detection and identification. Taxonomic delimitation of species, hybrid formulas and nomenclature of all taxa follow Wiegleb & Kaplan (1998), unless a more recent literature source is cited.



Fig. 1. – *Potamogeton x nerviger* sampled from the Verknė river 1 km south of Lielionys, at the village of Stakliškės, Lithuania; 24 Jul 2008. Photo J. Zalewska-Galosz.

Table 1. – Polymorphic positions in the alignment of ITS1 sequences of clones from the type collection of *Potamogeton xnerviger*, sequences from direct sequencing of morphologically matching plants recently collected at the type locality (?*P. xnerviger* dir. seq.) and of possible parental species. * – presence and absence of a single base in an additive pattern. Polymorphic nucleotide sites are coded using the IUPAC code.

Taxon	GB accession number	Position in the alignment																		
		22	23	25	37	48–54	55	58	60	69	78	87	94	98–99	106	110	124	156	158	178
<i>P. alpinus</i>	FJ883580	T	A	C	A	-----	A	A	C	G	T	A	C	--	C	C	G	T	C	G
<i>P. crispus</i>	FJ883590	C	A	C	T	-----	A	G	A	G	T	A	C	--	T	C	G	T	C	A
<i>P. gramineus</i>	FJ883587	T	A	C	A	-----	A	G	A	G	T	A	C	--	T	C	G	T	C	G
<i>P. lucens</i>	FJ883592	T	A	C	A	-----	A	G	A	G	T	A	C	--	T	C	G	T	C	G
<i>P. polygonifolius</i>	JX012087	T	C	C	A	CCTCTCG	A	G	A	G	C	A	C	AG	T	T	A	G	C	G
<i>P. praelongus</i>	JX012091	T	A	C	A	-----	A	G	A	G	T	A	C	--	T	C	G	T	C	G
<i>P. natans</i>	JX012090	T	A	C	A	-----	A	G	G	G	T	A	C	--	T	C	G	T	C	G
<i>P. perfoliatus</i>	FJ883583	T	A	T	A	-----	T	C	A	T	T	T	T	--	C	C	G	T	T	T
<i>P. nodosus</i>	FJ883594	T	A	T	A	-----	A	C	A	T	T	A	C	--	C	C	G	T	C	T
? <i>P. xnerviger</i> dir. seq.	MG432828	T	A	T	A	-----	W	C	A	T	T	W	Y	--	C	C	G	T	Y	T
<i>P. xnerviger</i> clone 1-15	MG432920	T	A	T	A	-----	T	C	A	T	T	T	T	--	C	C	G	T	T	T
<i>P. xnerviger</i> clone 1-1	MG432922	T	A	T	A	-----	T	C	A	T	T	T	T	--	C	C	G	T	C	G
<i>P. xnerviger</i> clone 1-4	MG432925	T	A	T	A	-----	T	C	A	T	T	A	C	--	C	C	G	T	C	G
<i>P. xnerviger</i> clone 1-6	MG432927	T	A	T	A	-----	A	C	A	T	T	A	C	--	C	C	G	T	C	G
<i>P. xnerviger</i> clone 1-16	MG432926	T	A	T	A	-----	A	C	A	T	T	A	C	--	C	C	G	T	C	G
<i>P. xnerviger</i> clone 1-10	MG432923	T	A	C	A	-----	A	G	A	G	T	A	C	--	C	C	G	T	C	G
<i>P. xnerviger</i> clone 1-20	MG432924	T	A	C	A	-----	A	C	A	T	T	A	C	--	C	C	G	T	C	T
<i>P. xnerviger</i> clone 1-13	MG432921	T	A	C	A	-----	T	C	A	T	T	T	T	--	C	C	G	T	T	T

Taxon	GB accession number	Position in the alignment																							
		180	186	187	198	209	210	211	218	223	225	226	227	228	230	236	237	238	239	241	242	247	250	269	
<i>P. alpinus</i>	FJ883580	A	G	G	G	A	A	A	T	G	A	C	T	A	T	C	-	T	T	T	T	A	A	A	
<i>P. crispus</i>	FJ883590	A	G	G	C	A	T	A	T	T	T	T	A	T	T	C	-	T	T	G	A	A	G	A	
<i>P. gramineus</i>	FJ883587	A	G	T	G	A	T	A	T	G	A	C	T	A	T	C	-	C	T	G	A	A	G	A	
<i>P. lucens</i>	FJ883592	A	G	G	G	A	T	A	T	G	A	C	T	A	T	C	-	C	T	G	A	A	G	A	
<i>P. polygonifolius</i>	JX012087	C	T	G	A	T	T	A	C	G	A	C	T	T	A	C	-	T	C	G	A	C	A	A	
<i>P. praelongus</i>	JX012091	A	G	G	G	A	T	A	T	G	A	C	T	A	T	C	-	C	T	G	A	A	A	A	
<i>P. natans</i>	JX012090	A	G	G	G	A	T	G	T	T	A	C	T	A	T	C	-	C	T	G	A	A	G	A	
<i>P. perfoliatus</i>	FJ883583	A	G	G	G	A	T	A	T	G	A	C	T	A	T	C	-	T	T	G	A	A	A	A	
<i>P. nodosus</i>	FJ883594	A	G	G	G	T	T	A	T	G	A	C	T	A	T	C	T	T	T	G	A	A	A	A	
? <i>P. xnerviger</i> dir. seq.	MG432828	A	G	G	G	W	T	A	T	G	A	C	T	A	T	C	*	T	T	G	A	A	A	A	
<i>P. xnerviger</i> clone 1-15	MG432920	A	G	G	G	A	T	A	T	G	A	C	T	A	T	C	-	T	T	G	A	A	A	A	
<i>P. xnerviger</i> clone 1-1	MG432922	A	G	G	G	A	T	A	T	G	A	C	T	A	T	C	-	T	T	G	A	A	A	G	
<i>P. xnerviger</i> clone 1-4	MG432925	A	G	G	G	A	T	A	T	G	A	C	T	A	T	C	-	T	T	G	A	A	A	A	
<i>P. xnerviger</i> clone 1-6	MG432927	A	G	G	G	A	T	A	T	G	A	C	T	A	T	C	-	T	T	G	A	A	A	A	
<i>P. xnerviger</i> clone 1-16	MG432926	A	G	G	G	T	T	A	T	G	A	C	T	A	T	C	-	T	T	G	A	A	A	A	
<i>P. xnerviger</i> clone 1-10	MG432923	A	G	G	G	T	T	A	T	G	A	C	T	A	T	C	T	T	T	G	A	A	A	A	
<i>P. xnerviger</i> clone 1-20	MG432924	A	G	G	G	T	T	A	T	G	A	C	T	A	T	C	T	T	T	G	A	A	A	A	
<i>P. xnerviger</i> clone 1-13	MG432921	A	G	G	G	A	T	A	T	G	A	C	T	A	T	-	-	T	T	G	A	A	A	A	

Table 2. – Sequence variation in the *rpl32-trnL* chloroplast intergenic spacer of *Potamogeton xnerviger* recently collected at the type locality, and of possible parental species. Positions diagnostic for *P. nodosus* are marked in bold.

Taxon	GB accession number	Position in the alignment																			
		54	60	97	104	123–128	141	142	151	157	167	184	191	206	268	280	295	320	331	338	354
<i>P. crispus</i>	FJ883608	G	T	A	G	TAAGAT	A	G	G	C	T	C	G	C	T	A	G	G	A	G	C
<i>P. lucens</i>	FJ883618	G	C	A	G	TAAGAT	–	C	G	T	A	A	T	C	T	A	G	T	A	T	C
<i>P. gramineus</i>	FJ883617	G	C	G	G	TAAGAT	–	C	G	T	A	C	T	C	T	A	G	T	A	T	C
<i>P. perfoliatus</i>	FJ883601	A	C	A	A	TAAGAT	–	C	G	T	A	A	T	G	T	A	G	G	A	T	C
<i>P. xnerviger</i>	MG458302	A	C	A	G	TAAGAT	–	C	G	T	A	A	T	A	T	A	C	G	A	T	C
<i>P. nodosus</i>	FJ883620	A	C	A	G	TAAGAT	–	C	G	T	A	A	T	A	T	A	C	G	A	T	C
<i>P. praelongus</i>	JX012075	G	C	A	G	TAAGAT	A	G	C	T	A	A	T	C	T	A	G	G	A	T	T
<i>P. natans</i>	JX012078	G	C	A	G	TAAGAT	A	G	A	T	A	A	T	C	T	A	G	G	A	T	C
<i>P. alpinus</i>	FJ883616	G	C	A	G	TAAGAT	–	C	G	T	A	A	T	G	T	C	G	G	G	T	C
<i>P. polygonifolius</i>	JX012086	G	C	A	G	-----	–	C	G	T	A	A	T	C	C	A	G	G	A	T	C

Taxon	GB accession number	Position in the alignment														
		359	364	367	368–371	426	430–443	454	459	463	516	529	570	575	576	580–586
<i>P. crispus</i>	FJ883608	C	A	A	AAAA	A	-----	T	T	G	T	C	T	A	T	TTATAAA
<i>P. lucens</i>	FJ883618	C	A	A	AAAA	A	-----	A	T	A	C	C	A	A	T	TTATAAA
<i>P. gramineus</i>	FJ883617	C	A	A	----	A	-----	A	T	A	C	C	A	A	T	TTATAAA
<i>P. perfoliatus</i>	FJ883601	C	A	A	----	A	-----	A	T	G	C	T	A	C	T	TTATAAA
<i>P. xnerviger</i>	MG458302	C	A	A	----	A	-----	A	T	G	C	C	A	A	T	TTATAAA
<i>P. nodosus</i>	FJ883620	C	A	A	----	A	-----	A	T	G	C	C	A	A	T	TTATAAA
<i>P. praelongus</i>	JX012075	C	A	T	AGAA	A	-----	G	T	G	C	C	A	A	G	TTATAAA
<i>P. natans</i>	JX012078	T	G	T	AGAA	T	-----ATATATTT	C	T	G	C	C	A	A	G	TTATAAA
<i>P. alpinus</i>	FJ883616	C	A	A	AAAA	A	TGTTTTATATATTT	A	G	G	C	C	A	A	T	TTATAAA
<i>P. polygonifolius</i>	JX012086	C	A	A	AAAA	A	-----	A	T	G	C	C	A	C	T	TTATAAA

Taxon	GB accession number	Position in the alignment													
		589	591	595	603	604	605	606	607–623	635	640	641	664	671	683–688
<i>P. crispus</i>	FJ883608	A	T	T	T	T	T	T	-----	T	T	A	T	G	-----
<i>P. lucens</i>	FJ883618	T	T	G	G	C	T	T	-----	T	T	T	T	G	TTAGAA
<i>P. gramineus</i>	FJ883617	T	T	G	T	C	T	T	-----	T	T	T	T	G	TTAGAA
<i>P. perfoliatus</i>	FJ883601	T	T	G	T	C	T	T	-----	A	T	A	T	G	TTAGAA
<i>P. xnerviger</i>	MG458302	T	T	G	T	C	T	T	-----	T	A	A	T	G	TTAGAA
<i>P. nodosus</i>	FJ883620	T	T	G	T	C	T	T	-----	T	A	A	T	G	TTAGAA
<i>P. praelongus</i>	JX012075	T	G	G	T	C	A	T	ATTAGATAAAATTCCTT	T	T	A	T	T	-----
<i>P. natans</i>	JX012078	T	T	G	T	C	T	G	-----	T	T	T	A/T	A	-----
<i>P. alpinus</i>	FJ883616	T	T	G	T	C	T	T	-----	A	T	T	T	G	TTCTAA
<i>P. polygonifolius</i>	JX012086	T	T	G	T	C	T	T	-----	–	T	T	T	A	-----



Fig. 2. – *Potamogeton ×nerviger* from the Verkné river cultivated in the Experimental garden of the Institute of Botany, Průhonice, Czech Republic. Photo Z. Kaplan.

DNA isolation, PCR amplification, sequencing and cloning

Leaf tissue taken from the type specimen of *P. ×nerviger* was ground to a fine powder using a Mixer Mill 400 (Retsch) and 3 mm tungsten beads. Total genomic DNA was extracted using the Plant & Fungi DNA Purification Kit (EURx) according to the manufacturer's protocol. The DNA template visualised in the agarose gel contained mainly fragments shorter than 300 bp. It is not surprising, therefore, that all attempts to amplify the entire nuclear ribosomal ITS region (ITS1, 5.8S gene, ITS2) as well as the *rpl32-trnL* intergenic spacer, despite using internal primers, failed. The only successful amplification was for ITS1 using the primers ITS A and ITS C (Blattner 1999). The following reaction mix composition was applied in a total volume of 25 μ l: 1 \times concentration of Taq Buffer with KCl (Thermo Scientific), 2 mM Mg^{2+} , 0.12 mM of each dNTP (Thermo Scientific), 0.2 μ M of each primer, 2 μ g of bovine serum albumin (BSA), 1 U of the Taq DNA Polymerase (Thermo Scientific) and 1 μ l of DNA template. A touchdown cycling profile was applied, including 5 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 62.5°C (with a decrease of 0.5°C per cycle and a constant temperature of 48°C starting from cycle 31) and 1 min at 72°C, and a final extension step of 10 min at 72°C. The ITS1 region was cloned to verify its additivity pattern following the method described by Zalewska-Gałosz et al. (2015). Twenty clones were checked by colony PCR. Five clones gave no product, the length of two others were different than expected. All of them were

excluded from the next steps. Thirteen clones contained right size inserts, from these eight were randomly selected and sequenced. Substitutions in cloned sequences that were not known from any of the *Potamogeton* species were considered as polymerase errors and corrected (0–2 sites per clone) before analysis and submission of the clones to GenBank. Each steps of DNA extraction and PCR reactions were carried out under the laminar chamber and using pipette filter tips to avoid possible contamination.

DNA isolation from the newly collected plants from the Verkné river, PCR amplification of the nuclear ribosomal ITS (ITS1, 5.8S gene, ITS2) region and chloroplast intergenic spacer *rpl32-trnL*, as well as direct sequencing of amplified fragments were performed as described in earlier *Potamogeton* studies (Zalewska-Gałosz et al. 2009, Zalewska-Gałosz & Ronikier 2011). Consensus sequences from two forward and reverse strands were manually edited and submitted to GenBank.

Sequences of studied regions were aligned in BIOEDIT v.7.0.5. (Hall 1999). Comparison of polymorphisms accumulated in ITS was done based on ITS1 because only ITS1 clones were available for *P. xnerviger* type (314–316 bp long).

Phenogram grouping of the most similar sequences was generated using the Neighbor Joining method computed in MEGA7 software (Kumar et al. 2016). The optimal tree with the sum of branch length = 44.90136719 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method (Nei & Kumar 2000) and are in the units of the number of base differences per sequence. The analysis involved 17 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 325 positions in the final dataset.

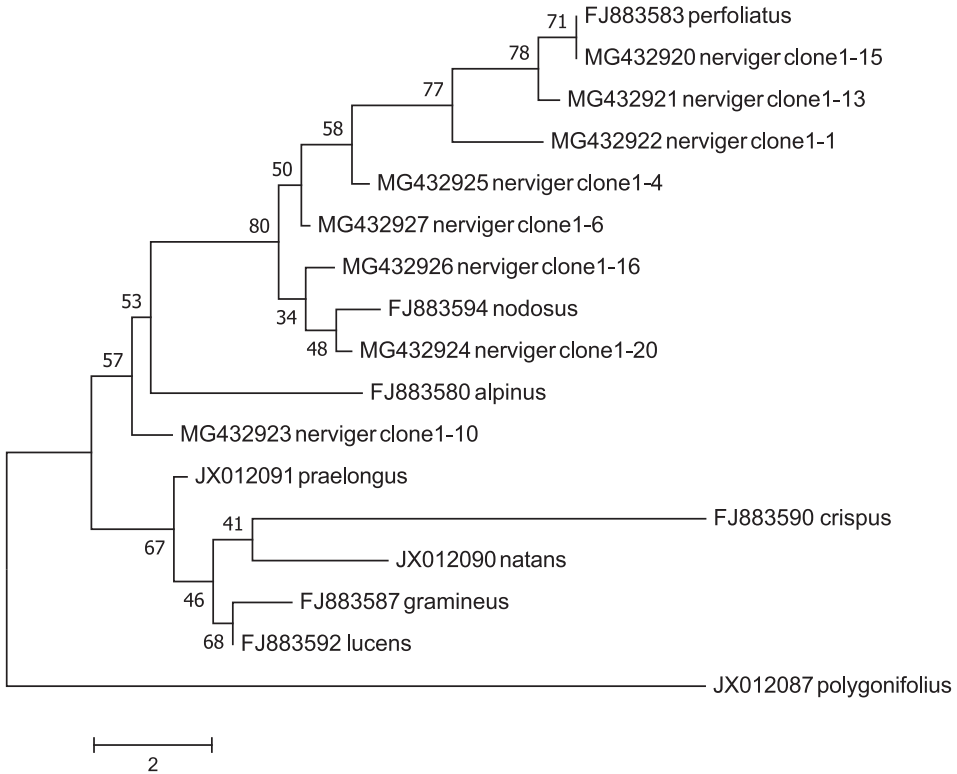
Analysis of stem anatomy

Anatomical features were assessed in a few mature specimens corresponding morphologically to *P. xnerviger* from its type locality. Short pieces were cut from the middle of the internode of the main stem. Stem samples were soaked in water for a few minutes. Approximately 0.05 mm thick slices of the stem were cut transversally with a razor blade under a stereomicroscope and then stained in an aqueous solution of toluidine blue for 1–3 minutes. Stained tissue was subsequently washed in distilled water. Stem anatomy was investigated using a transmitted-light microscope at a magnification of $\times 50$ (general anatomical pattern) or up to $\times 400$ (detailed view).

Results

Variation in ITS1 and analysis of the hybrid individuals

The ITS1 alignment prepared for the broad-leaved *Potamogeton* species, the putative hybrid from the Verkné river and the clones obtained from the *P. xnerviger* type was 325 bp long and covered the whole ITS1 region and the beginning of gene 5.8S. Forty-two polymorphisms were detected in the whole data set, thirty nine single nucleotide substitutions (SNP) and three insertions/deletions (indels), from one to seven bp long (Table 1). All *Potamogeton* species considered as potential parental taxa were clearly differentiated



based on species-specific polymorphisms and their composition. The ITS1 sequence obtained from the plant collected in the Verkné river displayed an additive polymorphism at five positions. Additionally, starting from position 237 in the alignment, the chromatogram consisted of double peaks caused by a shift between the ribotypes inherited from the parents due to the presence of an indel in one of them. The comparative analysis of the ITS1 sequence obtained from the putative *P. xnerviger* newly gathered in the Verkné river with the potential parental broad-leaved *Potamogeton* species allowed an unambiguous designation of the two parental taxa as *P. nodosus* and *P. perfoliatus*, while all other species can be ruled out as parents. The hybrid plant presented a rigorously additive sequence pattern of these two species and displayed no polymorphisms in other sites of their sequences. For *P. perfoliatus* the diagnostic positions were 55, 87, 94 and 158 of the alignment, while for *P. nodosus* the diagnostic position was 237, where this species possessed a species-specific indel (Table 1).

The chromatogram of ITS1 obtained by direct sequencing from the type of *P. xnerviger* had baseline noise, which was probably caused by poor quality of the DNA template

(A260/A280 ratio was 1.66) and could lead to misinterpretation; therefore, cloning of the ITS1 sequence was performed. Eight clones were sequenced, each of them 320-base-pairs long.

As shown in Fig. 3 and Table 1, three *P. xnerviger* clones were identical or nearly identical with sequences of two *Potamogeton* species. Clone 1-15 was identical and clone 1-13 nearly identical with the ITS1 sequence of *P. perfoliatus* while clone 1-20 was nearly identical (one different substitution) with the ITS1 sequence of *P. nodosus*. The rest five clones did not show close similarity to any of *Potamogeton* species. At least three of them (1-4, 1-6, 1-10), were probably PCR chimeras what was reflected in their basal position in the tree branches (Fig. 3). Therefore, based on the evidence provided, all *Potamogeton* species can be excluded as parental species of *P. xnerviger* except for *P. perfoliatus* and *P. nodosus*.

Variation in the cpDNA region and identification of the maternal parent

The sequences of the *rpl32-trnL* intergenic spacer were 632–690 base pairs long and their alignment 690 bp. The whole data set aligned for broad-leaved *Potamogeton* taxa was highly polymorphic, and 49 polymorphic sites were identified. This included 43 single nucleotide polymorphisms (SNP) and six insertions/deletions one to 17 bp long (Table 2). The parental species of the hybrid, *P. nodosus* and *P. perfoliatus*, differ by seven SNPs (104, 206, 295, 529, 575, 635 and 640 of the alignment), of which three are diagnostic for *P. nodosus* (Table 2). The *rpl32-trnL* sequence of the newly collected hybrid *P. nodosus* × *P. perfoliatus* from the Verkné river was identical to that of *P. nodosus*. Because *Potamogeton* hybrids were demonstrated to inherit cpDNA maternally (Kaplan & Fehrer 2006), *P. nodosus* is proved to be the maternal parent of the hybrid studied.

Stem anatomy

Potamogeton hybrid individuals recently collected from the Verkné river had stele of trio type, endodermis of O-type (with the cell wall thickening often indistinct) and lacked pseudohypodermis and the subepidermal and cortical strands.

Discussion

Successful DNA analysis of the historical type specimen

Herbarium collections, in which many plant specimens have been gathered over the years, are potentially a very useful source of molecular information, especially for endemic, rare or even extinct taxa (e.g. Drábková et al. 2002). However, as it is clear from our study, herbarium material is often problematic for molecular analysis and cannot yield some kinds of information. In general, DNA samples obtained from herbarium specimens are more degraded than those from fresh material. There is some evidence that the quality of DNA decreases with increased age of herbarium specimens. However, other factors, such as the way of drying, conditions of preservation or disinfection methods of the herbarium collections, probably have greater effects on the quality of DNA.

Despite our repeated efforts and various modifications of the reaction conditions, the only sequence we succeeded in amplifying from the 188-year-old type specimen of

Potamogeton ×nerviger was ITS1. The efforts described here represent the second time that it was attempted to obtain molecular information from an old herbarium specimen of *Potamogeton*. Previously, amplification of ITS1 was successfully performed from a 115-year-old herbarium specimen of *P. ×subrufus*, which allowed identification of the parental species of this hybrid (Zalewska-Gałosz & Kwolek 2014). During cloning procedure of ITS1 of *P. ×nerviger* some problems appeared. For eight, randomly selected and sequenced clones at least three of them were PCR chimeras (Fig. 3). Recombinant clones can occur in PCR cloning procedure of hybrids, especially dealing with degraded DNA, however, these chimeras as PCR artefacts should be excluded from the next steps of analysis. Two positions in the alignment, where the same substitutions are repeated (position 25 in clones: 1-10, 1-20, 1-13 and position 178 in clones: 1-1, 1-4, 1-6, 1-16 and 1-10; Table 1) are difficult to explain and suggest some real variation. Due to precautions applied and the fact that it was the first analysis of *Potamogeton* sample in the laboratory, the risk of contamination during PCR procedure was very low, however cannot be completely ruled out. Despite problems mentioned above, ITS1 fragment provided sufficient evidence for the hybrid origin of *P. ×nerviger*. Based on the species-specific polymorphism detected in the clones of the ribotype variants of ITS1 obtained from *P. ×nerviger*, we were able to identify its parental species, namely *P. perfoliatus* and *P. nodosus*, in this study. The same parentage was detected in plants collected at the type locality. However, in this analysis, the molecular information gathered was wider and comprised the whole ITS region and *rpl32-trnL* intergenic spacer. In addition to the molecular data, the anatomical characters of the individuals of *P. ×nerviger* recently collected at the type locality were in accordance with those expressed by parental taxa and the hybrid *P. perfoliatus* × *P. nodosus* (Zalewska-Gałosz et al. 2010).

History of the hybrid in the Verknė river

It has been demonstrated that *Potamogeton* hybrids, although the great majority are sterile (e.g. Hagström 1916, Preston 1995, Wiegleb & Kaplan 1998, Kaplan 2007, Kaplan et al. 2009), are able to persist at their sites for over hundreds of years due to vegetative propagation (e.g. Preston et al. 1998, King et al. 2001, Kaplan & Fehrer 2007, 2013, Zalewska-Gałosz 2010). Our observations indicate that the colony of plants now growing at the type locality of *P. ×nerviger* is a clone spreading vegetatively and persisting there for approximately two centuries. The fact that many duplicates of the type collection were gathered at the type locality in the 1820s (see Kaplan & Zalewska-Gałosz 2004) suggests that already at that time the hybrid clone was very abundant there, indicating its history reaches back much farther.

Distribution of the hybrid Potamogeton nodosus × P. perfoliatus

The existence of the hybrid *P. nodosus* × *P. perfoliatus* was revealed only recently (Zalewska-Gałosz et al. 2010). To date it has been recorded in Poland, Lithuania, Montenegro, Sudan, Niger and Madagascar (Zalewska-Gałosz et al. 2010, Kaplan et al. 2013).

Potamogeton ×nerviger, with the assumed parentage *P. alpinus* × *P. lucens*, has been reported from Ireland (Dandy 1975, Preston 1995), Germany (Wiegleb et al. 2008), Estonia (Trei et al. 2003), Lithuania (Fischer 1907, Trei et al. 2003) and Russia (Papčėnkov

2007). However, the existence of the hybrid *P. alpinus* × *P. lucens* has never been proved by molecular analysis. Sequencing of plants from Krüselinsee in Germany recorded as *P. ×nerviger* by Wiegand et al. (2008) showed that it was actually a slender form of *P. ×salicifolius*, i.e. the hybrid *P. lucens* × *P. perfoliatus* (Kaplan & Fehrer 2011). Considering the fact that none of the analysed populations hitherto ascribed to *P. ×nerviger* proved to be *P. alpinus* × *P. lucens*, we consider this hybrid combination as doubtful and molecular confirmation of the identity of the other populations as desirable.

Nomenclatural consequences

Our study shows that the type collection of *P. ×nerviger* is not *P. alpinus* × *P. lucens*, as has been widely believed, but another hybrid, *P. nodosus* × *P. perfoliatus*, which had already been named *P. ×assidens* (Zalewska-Gałosz et al. 2010). As we have now shown that the type of *P. ×assidens* and that of *P. ×nerviger* belong to the same nothospecies, under the priority rule of the International Code of Nomenclature, the latter name, as the earlier validly published and legitimate name for the hybrid *P. nodosus* × *P. perfoliatus*, should now replace *P. ×assidens*. However, the name *P. ×nerviger*, although published 190 years ago and well established in the literature, has never been interpreted in this sense. Consequently, this nomenclatural change would cause considerable confusion and usage of this name would be inevitably associated with ambiguity as to the actual parentage of the taxon to which it refers. Replacing the taxonomically clear name *P. ×assidens* by the controversial name *P. ×nerviger* would constitute an undesirable and disadvantageous change for purely formal reasons. To avoid confusion of literature records and to ensure nomenclatural stability, the name *P. ×nerviger* is proposed for rejection (Kaplan & Zalewska-Gałosz 2018).

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Souhrn

Velkou část diverzity rodu *Potamogeton* tvoří kříženci. Jejich přesná taxonomická příslušnost a rozšíření jsou však stále nedostatečně známy. Příkladem je *P. ×nerviger*, který byl popsán v ranku druhu z Litvy v roce 1827. Na základě morfologických a anatomických znaků ho různí autoři chápali jako jeden z fenotypů podobných druhů nebo jako křížence různých rodičovských kombinací. V posledních desetiletích byl považován za křížence *P. alpinus* × *P. lucens*. Původní herbářový materiál *P. ×nerviger* a rostliny nyní sebrané na typové lokalitě jsme podrobili molekulárnímu, morfologickému a anatomickému zkoumání. Ze 188 let staré typové položky jsme úspěšně amplifikovali a osekvenovali úsek ITS1 jaderné ribosomální DNA. Sekvence byly identické se sekvencemi získanými z morfologicky podobných rostlin nalezených na typové lokalitě. Porovnání s molekulárními znaky spektra možných rodičovských druhů odhalilo, že *P. ×nerviger* není křížence druhů *P. alpinus* a *P. lucens*, jak se dosud předpokládalo, ale *P. nodosus* × *P. perfoliatus*, pro něhož se nyní používá jméno *P. ×assidens*. Identifikaci založenou na molekulárních datech podporují také znaky v anatomii lodyhy. Naproti tomu skutečná existence dříve uváděného křížence *P. alpinus* × *P. lucens* je pochybná. Diskutovány jsou také důsledky pro nomenklaturu a interpretaci údajů z jiných území.

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