

Discovery of a new, recurrently formed *Potamogeton* hybrid in Europe and Africa: Molecular evidence and morphological comparison of different clones

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Abstract A new *Potamogeton* hybrid resulting from crossing between *P. nodosus* and *P. perfoliatus*, and occurring in Europe and Africa is described here as *P. ×assidens*. The hybrid identity was unequivocally confirmed by molecular study of ITS and selected chloroplast DNA regions. In European populations, for which the maternal taxon was identified based on cpDNA as *P. nodosus*, maternally driven expression of characters may account to a large degree for shaping the range of morphological variability of the hybrid taxon. This was accompanied by a matroclinal concerted evolution observed at the molecular level in the ITS sequences. Our observations may suggest the presence of some genetic mechanisms that promote a higher impact of the maternal lineage on the expression and evolution of the hybrid variability both at the molecular (direction of concerted evolution in hybrids) and the morphological level. Distinctive characters of *P. ×assidens* and other morphologically close *Potamogeton* hybrids are discussed. The hybrid most similar to *P. ×assidens*, namely *P. ×rectifolius*, is typified.

Keywords Africa; Europe; hybrids; ITS; matroclinal concerted evolution; matroclinal variation; *rpl32-trnL* intergenic spacer; *rps12-rpl20* intergenic spacer; taxonomy

■ INTRODUCTION

Interspecific hybridisation is an important source of plant diversity (Stace 1975). In some cases homoploid hybridisation can result in formation of stabilised species (Ungerer & al., 1998). More often, however, reticulation can lead to creation of stable hybrid zones (Barton & Hewitt, 1985), where besides selection against unfit hybrid genotypes, also introgression and gene flow from one species to another can take place. In the genus *Potamogeton* L. hybridisation processes are facilitated by a high frequency of sympatric species due to their environmental requirements restricted to aquatic habitats, and often lead to the emergence of easily recognisable biological entities (Wiegleb & Kaplan, 1998). The hybrids are mostly sterile, but often create stable and long-persistent populations of high ecological significance by means of vegetative propagation (Preston, 1995). Some of the hybrid taxa, as *P. ×nitens* (= *P. gramineus* × *P. perfoliatus*), *P. ×angustifolius* (= *P. gramineus* × *P. lucens*) or *P. ×salicifolius* (= *P. lucens* × *P. perfoliatus*; for authors of plant names see Wiegleb & Kaplan, 1998) are relatively frequent (Dandy, 1975) and may occupy ecological niches not accessible for parental species, especially in rivers. Obviously, hybrid formation and evolution have a potential to occur repeatedly at different times and geographical locations, which may result in various degrees of morphological and genetic differences in the offspring of the same parental taxa (Hegarty & Hiscock, 2004). It is therefore particularly interesting to survey the expression of features in widely distributed populations of hybrid taxa originating from presumably independent and recurrent hybridisation events. Such studies may bring insights

into evolutionary processes connected with hybrid formation and into taxonomical approaches dealing with classification of hybrid taxa.

As taxonomically and ecologically important entities, *Potamogeton* hybrids have drawn attention of biologists for a long time. Especially in Europe, thanks to such researchers as A. Fryer, A. Bennett, G. Fischer, the outstanding *Potamogeton* taxonomist J.O. Hagström and—in the second half of the 20th century—J.E. Dandy and G. Taylor, the knowledge about the existence and diversity of hybrid taxa seems to be almost complete. Currently, 35 interspecific hybrids are recognised, involving 21 *Potamogeton* species out of the total of 22 species occurring in Europe (numbers estimated based on compiled literature survey). The majority of hybrids was described by 1916 and only five further taxa were added in the second half of the 20th century, all based solely on morphological and anatomical characters. In recent years, biochemical and molecular tools commonly used in taxonomical studies have brought about substantial new facts to our knowledge on hybridisation in *Potamogeton*. Occurrence of a triple *Potamogeton* hybrid was demonstrated (Kaplan & Fehrer, 2007) and a cryptic linear-leaved hybrid between morphologically similar species was recognised (Whittall & al., 2004). Biparental inheritance has also been examined repeatedly using analysis of ribosomal ITS (Internal Transcribed Spacer) in hybrid taxa of *Potamogeton* (King & al., 2001; Kaplan & Fehrer, 2004, 2006, 2007; Zalewska-Gałosz & al., 2009). A well developed framework for assessment of morphology and molecular variation is available for further detailed studies and, indeed, new and intriguing research perspectives in *Potamogeton* are still far from being exhausted.

During field investigation of aquatic ecosystems of Drawieński National Park (NW Poland), an interesting broad-leaved *Potamogeton* hybrid was found in the middle part of the Drawa River (Kraska & al., 2004). Plants were heterophyllous, with sessile submerged leaves. They partly resembled *P. alpinus* in habit but their acute and denticulate submerged leaves and branched stems clearly precluded such identification. Based on morphological analysis the plants were identified as *P. ×nericius*, the hybrid between *P. alpinus* and *P. gramineus* (Zalewska-Galosz, 2002). In 2006 a new locality of this hybrid was recorded in the Rospuda River (NE Poland). This new discovery provided an impetus for a careful examination of morphological and anatomical characters as well as a re-evaluation of plants from the Drawa River population, collected in 2004–2005. Surprisingly, stem anatomy investigations, which had not been carried out before, revealed a lack of inherited *P. gramineus* characters in the putative hybrid. Therefore, because some doubts in the validity of the determination appeared, we decided to apply molecular studies to support or reject the original identification.

In a parallel study, a morphologically similar hybrid has been discovered in herbarium material from Africa investigated for a revision of broad-leaved African species (see Kaplan & Symoens, 2004, 2005). The specimens discovered were characterized by a combination of characters absent in any *Potamogeton* known from Africa, as well as from the surrounding islands or the adjacent part of Europe. In particular, the combination of sessile submerged leaves with semiamplexicaul bases and the higher number of veins indicated their possible hybrid origin with *P. perfoliatus* as one of the parents. The occurrence of floating leaves in most of the clones also suggested the involvement of a species with the capacity to develop such leaves. The only suitable candidate among African *Potamogeton* species is *P. nodosus*.

A detailed study of variation focused on these Polish and African materials has been carried out with the following successive aims: (1) to analyse these morphologically unusual *Potamogeton* specimens and investigate their morphological and anatomical features in order to identify probable parental taxa; (2) to examine molecular characteristics of these putative hybrids and correlate them with morphological evidence; (3) to contribute to the knowledge on diversity and variation of hybrid plants by evaluating the morphological differences between our European and African materials and to discuss possible sources of divergence; (4) to resolve the taxonomic status of these plants based on combined data.

■ MATERIALS AND METHODS

Morphological study. — Morphological studies on the Polish hybrids were based on herbarium specimens collected in the Drawa River in 2003 and 2004 and in the Rospuda River in 2006. A total of 50 vouchers was collected, deposited mainly in KRA, and partly in KRAM and PRA. Morphological characters of stem, submerged, transitional and floating leaves, stipules, inflorescence, peduncles and flowers were measured

or qualitatively described. For each of 50 individuals up to 55 features were examined (Appendix 1). Similar observations were made also on the African material, with the limitations given by the small number of available herbarium specimens (23 plants from 5 sites).

Analysis of stem anatomy. — Anatomical features were assessed in all specimens collected. Short pieces of stem were cut from the middle of the internode of the main stem. Samples of several decades old African material were first soaked for a few days in a solution of equal parts of water, ethanol and glycerol. Samples from recently collected Polish material were only soaked in water for a few minutes. Approximately 0.05 mm thick slices of stem were cut transversally with a razor blade under a stereomicroscope and then stained in aquatic 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).

Sampling of material for molecular analysis. — All samples included in the molecular analysis were collected from fresh material in the field and stored in plastic scintillation tubes with silica gel. Samples of putative hybrid plants were collected from two populations in Poland. Taking into account the presumably clonal population structure and relatively wide distribution of the populations along both rivers, ramets were sampled at a distance of at least 5 m between plant clumps to increase the probability of collecting genetically different individuals.

As comparative material, samples of all potential parental species from several regional populations were included in the analysis (Appendix 2). Voucher specimens for all sequenced plants are deposited in KRA. The African material could not be included in the analysis because only old herbarium specimens were available and previous attempts to extract sufficiently well-preserved DNA from old herbarium material of *Potamogeton* were unsuccessful.

DNA isolation, PCR amplification and sequencing. — The amount of 10–15 mg of dried plant material was used for DNA isolation. The plant tissue was ground to fine powder using Mixer Mill 200 (Retsch) and 3 mm tungsten beads. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's protocol (the final elution step was carried out using $2 \times 50 \mu\text{l}$ elution buffer). Quality of DNA extractions was verified by electrophoresis on 1% agarose gels.

The nuclear ribosomal Internal Transcribed Spacer region (including ITS1, 5.8S and ITS2) was amplified using the primers ITS1A and ITS 4 (White & al., 1990; Fuertes Aguilar & al., 1999). The following reaction mix composition was applied in a total volume of 25 μl : $1 \times$ concentration of PCR AmpliTaq Buffer (Applied Biosystems), 2.5 mM Mg^{2+} , 0.11 mM of each dNTP (Roche Diagnostics), 0.2 μM of each primer, 1 μg of bovine serum albumine (BSA), 1 U of the AmpliTaq DNA Polymerase (Applied Biosystems) and 0.5 μl of DNA template. A touchdown cycling profile was applied, including 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 56°C (with a decrease of 0.5°C per cycle and a constant temperature of 48°C starting from cycle 15) and 1 min at 72°C, and a final extension step of 10 min at 72°C.

Two chloroplast (cpDNA) regions were analysed in all sampled taxa. The *rps12-rpl20* intergenic spacer (modified from Hamilton, 1999 according to Shaw & al., 2005) was amplified in all samples as it previously appeared useful in discerning between several *Potamogeton* species (Kaplan & Fehrer, 2007). This region did not differentiate all species studied, therefore, in a second step the *rpl32-trnL* intergenic spacer, reported by Shaw & al. (2007) to be highly variable, was amplified in single samples per taxon. The same reaction mix composition as described above was used, except for primer concentrations which were 0.1 μ M. The PCR cycling conditions were: 5 min at 80°C, 30 cycles of 1 min at 95°C, 1 min at 50°C, ramp of 0.3°C/s to 65°C and 4 min at 65°C, followed by a final extension step of 5 min at 65°C. PCR reactions were performed in a GeneAmp 9700 thermal cycler (Applied Biosystems) or a PTC200 thermal cycler (MJ Research).

PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) according to the manufacturer's protocol and sequenced in two directions using the primers used for amplification. Sequencing was performed using BigDye Terminator v.3.1 (Applied Biosystems) with supplied 5 \times sequencing buffer, according to the manufacturer's manual. Sequencing products were purified using the Ethanol/EDTA protocol, resuspended in 12 μ l formamide and separated on an ABI 3100-Avant Genetic Analyser using 50 cm capillaries and POP-6 polymer (Applied Biosystems). Raw sequencing profiles were analysed with the DNA Sequencing Analysis Software v.5.1 (Applied Biosystems).

The sequences were manually verified/adjusted using the software Finch TV v.1.4.0 (Geospiza Inc.). Alignments of sequences for all regions were conducted manually using BIOEDIT v.5.0.9. (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Additive nucleotide polymorphisms were examined with two strands to ensure their consistency and coded using the IUPAC nucleotide ambiguity codes. The DNA sequences are deposited at GenBank (Appendix 2).

■ RESULTS

Morphological description of the hybrid. — *Stem* up to 1.5 m long, terete, unbranched or sparingly branched above. *Submerged leaves* sessile, 48–120–140(–170) mm long, (11–)14–22(–26) mm wide, (5–)7–9(–10) times as long as wide, translucent, narrowly elliptical to obovate, bright green, with reddish or brownish tinge when dried, gradually tapering to cuneate or rounded, often semiamplexicaul base, acuminate, acute, subacute or obtuse to rounded and sometimes slightly but distinctly hooded at apex, denticulate at margins (or minute and deciduous denticles not preserved in specimens), midrib bordered by band of apex reaching lacunae, lateral veins (4–)6–8(–10) on each side, 2(–3) of them stronger because bordered by band of lacunae, these along outermost stronger veins reaching 2/3 of leaf length, the stronger veins alternating with weaker ones, secondary veins numerous, more or less ascending, irregular. *Transitional leaves* similar to floating ones but with semi-opaque lamina. *Floating leaves* subsessile to petiolate, lamina 25–80 mm long, 12–23 mm wide, 2–4 times as long as wide, subcoriaceous, opaque, oblong

to elliptical or narrowly obovate, green, brownish when dry, gradually tapering to base, subacute, obtuse to rounded at apex, lateral veins 6–10 on each side, secondary veins numerous, ascending in centre of leaf and transverse towards the margin, petioles 10–35 mm long, shorter than lamina. *Stipules* 8–40 mm long, open, translucent and hyaline, greyish to brown when dry, rounded at apex, fugacious, present only on youngest leaves and disappearing early, veins inconspicuous when dry, 2 of them slightly stronger than others. *Inflorescence* (7–)10–28 mm long. *Peduncles* 15–80(–97) mm long, terete, as thick as stem, of uniform diameter throughout length. *Flowers* in 6–11 whorls, more or less contiguous. *Fruits* not observed and presumably not produced (plants sterile). *Stem anatomy*: Stele trio type, endodermis O-type (cell wall thickening often indistinct), pseudohypodermis absent, subepidermal and cortical strands absent.

The African specimens of the putative hybrid are clearly intermediate between *P. nodosus* and *P. perfoliatus* in many characters including the shape and size of submerged leaves, the number of longitudinal veins and the shape of the leaf base. In particular, the semi-amplexicaul bases are clearly derived from *P. perfoliatus*, the only species occurring in Africa and characterized by amplexicaul leaves. This is an universal character of all *P. perfoliatus* hybrids (see, e.g., Dandy, 1975; Preston, 1995; Fant & Preston, 2004; Kaplan & Fehrer, 2004, 2006, 2007; Kaplan & Zalewska-Gałosz, 2004; Kaplan, 2005, 2007). The hybrid is also more similar to *P. perfoliatus* in its sessile leaves, the shape of the leaf apex, short persistence of stipules and size of the inflorescence. In contrast, the African hybrid differs from this parental species by its longer submerged leaves with fewer longitudinal veins, generally more flowers in the inflorescence, and—at least in the case of some clones—the capacity to produce floating leaves; these characters are derived from *P. nodosus*. The main character distinguishing the hybrid from *P. nodosus* can be found in submerged leaves, which are sessile, in contrast to petiolate submerged leaves of that species. In addition, the floating leaves have smaller laminae, the petioles are markedly shorter than those of *P. nodosus*, and the inflorescences are shorter as well. Both parental species differ from the investigated hybrid, which is consistently sterile, in their capacity to produce well-formed fruits. The hybrid shares with both parents the same pattern of stem anatomy, which is markedly different from that of all other broad-leaved *Potamogeton* species widespread in Africa (for details see Kaplan & Symoens, 2005). No known African species displays this combination of characters; similarly, it could not be attributed to potential crossing of any other pair of African species. All morphological and anatomical characters, when considered in conjunction, allow thus an unequivocal identification of the intermediate plants as *P. nodosus* \times *P. perfoliatus*.

In the case of the European plants studied, morphological and anatomical investigations were inconclusive regarding exact identification of parental taxa. Such identification was enabled by the subsequent molecular analysis.

Variation of ITS and analysis of the hybrid individuals. — The ITS sequences obtained from the studied samples had 623–626 bp. Sixty-four polymorphisms were detected in the total dataset, including 59 nucleotide substitutions and five

single-base pair insertions/deletions (indels; Table S1 in the Electronic Supplement to this article). All the analysed species were clearly differentiated based on the composition of polymorphisms and on their specific polymorphisms. All samples of each taxon had identical sequences and did not have any additive, intra-individual polymorphism, except for *P. nodosus*, where one accession had one intra-individual single nucleotide polymorphism (A/G) at alignment position 502, while other samples had A. All examined accessions of the putative hybrids from two Polish populations had identical sequences. They displayed an additive polymorphism at 18 sites: 17 single nucleotide polymorphisms and one single-base pair indel causing a shift in the overlapping ribotype sequences. The comparative analysis of hybrid sequence with those of putative parental taxa allowed an unambiguous designation of two parental taxa as *P. nodosus* and *P. perfoliatus* (Table S1 and Fig. S1 in the Electronic Supplement). The hybrid plants presented a rigorously additive sequence pattern of these two taxa and displayed no polymorphisms in other sites of their sequences. In the plants from Drawa River the double nucleotide peaks had nearly equal heights, while in the plants from Rospuda River the variants from *P. nodosus* dominated over those from *P. perfoliatus* with an average height ratio 5.2 : 1 (Fig. S1 in the Electronic Supplement).

Variation of cpDNA regions and identification of the female parental taxon. — The sequences of the *rps12-rpl20* intergenic spacer were 734–771 bp long. This cpDNA region was polymorphic among several taxa as reported by Kaplan & Fehrer (2006). It contained six single nucleotide polymorphisms and four insertions (duplications). The polymorphism was distributed among taxa and no intraspecific variation was observed. The *rps12-rpl20* sequences allowed distinguishing most species, however, they did not provide differentiating characters for the two parental species of the hybrid studied and therefore the direction of maternal inheritance could not be assessed based on this marker. The sequences of the *rpl32-trnL* intergenic spacer, used in the second step of the cpDNA analysis, had 732–758 bp. Among samples of all examined taxa, 23 polymorphic sites were identified. They included 19 single nucleotide substitutions and four insertions/deletions (indels): two A/T stretch length polymorphisms, and two duplications of 7 and 14 bp. All species had diagnostic polymorphisms and the parental taxa (*P. nodosus* and *P. perfoliatus*) differed by substitutions at positions 104 (A/G), 205 (A/G), 294 (C/G), 528 (C/T), 574 (A/C), 617 (A/T), 622 (A/T), and a 1 bp indel at position 743 of the alignment. This polymorphism allowed to unambiguously distinguish *P. nodosus* as the donor of cpDNA in both investigated hybrid populations (hybrids shared an identical sequence with this species). The level of polymorphism found in this intergenic spacer, compared to the other examined cpDNA region, confirms the utility of this region in analyses at intra- and interspecific level, as indicated by Shaw & al. (2007).

Description of the new hybrid taxon. — Morphological, anatomical and molecular features provide evidence that the investigated hybrid discovered in distant populations in Europe and Africa results from hybridisation between *P. perfoliatus* and *P. nodosus*. As this taxon has not been described yet, we describe it, based on our data, as a new hybrid according to ICBN rules.

Diagnostic features: submerged leaves sessile, with semi-amplexicaul bases, minutely denticulate at margin, the lateral veins (4)–6–8(–10) on each side, 2(–3) stronger, bordered by band of lacunae, lacunae along outermost stronger vein reaching 2/3 leaf length, stronger veins alternating with weaker ones, pseudohypodermis absent, cortical strands absent.

Potamogeton × *assidens* Z. Kaplan, Zalewska-Gałosz & M. Ronikier, **nothosp. nov.** (= *P. nodosus* × *P. perfoliatus*) (Fig. 1)

Diagnosis. — Planta hybrida, proprietatibus intermediis taxoni parentalibus i.e., *Potamogetonis nodosi* et *P. perfoliati*; a *P. nodoso* praecipue foliis submersis sessilibus, natantibus lamina minore petioloque distincte brevior et inflorescentia brevior; a *P. perfoliato* autem foliis submersis longioribus et foliis natantibus efficiendis; ab utroque parente sterilitate perpetua differt.

Type. — North-eastern POLAND, Suwalszczyzna (Pojezierze Suwalskie lakeland) region, Rospuda valley, in Rospuda River near Święte Miejsce (53°56'56.4" N 22°52'27.4" E), on sandy bottom, ca. 1.5 m deep; 21 July 2006, coll. J. Zalewska-Gałosz (holotype: KRA 343630; isotypes: KRA 350401, 350402, 350403, KRAM 564529, 564530, PRA 1449–1453).

Paratypes. — North-western POLAND, Western Pomerania, in the Drawa River, below station called Na Moczele

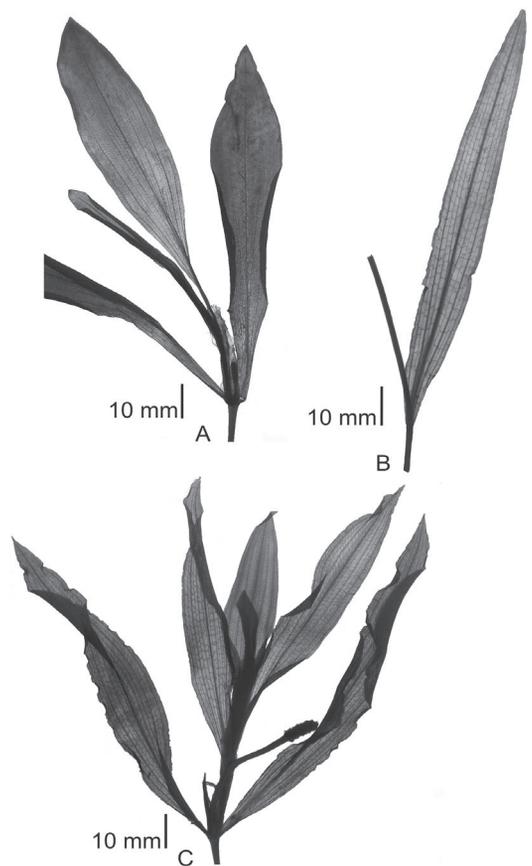


Fig. 1. *Potamogeton* × *assidens* from the Drawa River. **A**, the apical part with floating leaves; **B**, the submerged leaf from middle part of the stem; **C**, the apical part with inflorescence. Based on herbarium specimen KRA 0343629.

(53°04'50"N 15°55'30"E), on sandy or gravelly bottom at the depth of 0.4–1.4 m; 6 July 2004, coll. J. Zalewska-Gałosz (KRA 343629); SUDAN, about 15 km W of Wad Medani, El Gezira, in irrigated area, clay soil, in shallow water in a ditch, 11 Feb. 1965, coll. W.J.J.O. de Wilde & al. (Plantae Africae centralis exsiccatae no. 5734) (BR, K, P, WAG); SUDAN, Wad Medani, Gezira Scheme, in irrigated area, in shallow water of slowly running ditch, 10 Feb. 1965, coll. W.J.J.O. de Wilde & al. (Plantae Africae centralis exsiccatae no. 5712) (WAG); NIGER, Air Mts., Tamgak Wadi, 19°5'N, alt. 720 m, 27 March 1970, coll. P.N. Bradley 123 (K); MADAGASCAR, Centre Ouest, Ampasimbazimba sur la Mazy, eaux courantes, aux environs de sources thermales, alt. 800 m, aout 1912, coll. H. Perrier de la Bathie 7148 (P); MADAGASCAR, Ouest, W de Betroka, eaux calcaires, cipolins, alt. 700 m, VII [19]09, coll. H. Perrier de la Bathie 12689 (P).

Etymology. – The taxon's epithet refers to the sessile submerged leaves, one of the diagnostic features distinguishing the new hybrid from *P. nodosus*, which has petiolate submerged leaves.

Habitats of P. ×assidens. – In the Drawa River plants were found in an about 2 km long stretch of its middle part (53°04'50"N 15°55'30"E). Individuals formed small separated clumps or were dispersed in patches dominated by *P. pectinatus*, *P. ×sparganiiifolius* (= *P. gramineus* × *P. natans*) or *P. perfoliatus*. Submerged forms of *Butomus umbellatus* and *Sagittaria sagittifolia* were also common in the place. *Potamogeton ×assidens* grows on sandy or gravelly bottom at a depth of 0.4–1.4 m. Physiochemical properties of water in relevant part of the Drawa River are summarised by Kraska & al. (2004). The population of the second European locality is dispersed along an 8 km long section of the Rospuda River near Święte Miejsce. Individuals of *P. ×assidens* grow there on sandy bottom, in running water, at a depth of 0.4–1.5 m. They co-occurred in patches with another *P. nodosus* hybrid, namely *P. ×subobtusus* (= *P. alpinus* × *P. nodosus*; Zalewska-Gałosz & al., 2009). Other, locally dominant taxa were *P. pectinatus*, *P. perfoliatus*, *P. natans*, *Batrachium fluitans* and submerged phenotypes of *Butomus umbellatus* and *Sagittaria sagittifolia*. *Potamogeton nodosus* was not found in the close vicinity of any of these localities.

The habitats of the African populations are little known. As deduced from the data provided in herbarium labels, *P. ×assidens* grew there in shallow, standing or slowly running water, above clay bottom rich in calcium. Both parental species were documented at the same sites as the hybrid or at least they are known to occur in the same region.

DISCUSSION

Morphological variation across populations and expression of parental features in the hybrid individuals. — In contrast to a relatively complicated identification process of *P. ×assidens* in the Polish populations, the combined morphological and anatomical examination of the individuals from Africa was sufficient to identify the plants as hybrids between *P. nodosus* and *P. perfoliatus*. Unlike in Europe, this identification was facilitated by the co-occurrence of hybrids and their

parental taxa at some localities, as well as the generally low diversity of broad-leaved species of *Potamogeton* in Africa (Kaplan & Symoens, 2005).

All plants identified as *P. ×assidens* share a large set of characters, especially regarding individuals from Polish localities. In general, plants are sparingly branched, heterophyllous macrophytes with coriaceous floating leaves and well developed, oblong and denticulate submerged leaves with semiamplexicaul bases. The stipules are delicate, hyaline, early eroded to fibrous remnants. Besides these similarities, however, some differences have also been observed between Polish and African plants. First, some of the quantitative characters diverge, especially the length of submerged leaves, which are shorter in African specimens and longer in Polish plants. The main morphological difference, however, lies in differently shaped apices of submerged leaves. Individuals from Polish localities are characterized by acuminate or acute submerged leaves more closely resembling *P. nodosus* whereas African plants develop subacute, rounded or even slightly hooded leaves similar to *P. perfoliatus*. Such differences are striking but they are in agreement with the previous observations that character expression in hybrids is largely unpredictable. Moreover, one of possible processes largely extending the range of variation of a hybrid may involve matroclinal inheritance, due to which a hybrid may more closely resemble the female parent rather than being intermediate. This tendency can be clearly observed in the European plants, where hybrids are morphologically closer to *P. nodosus* and also possess cpDNA inherited from this species as evidenced by the molecular study. Due to a large geographic distance it can be assumed that the African plants resulted from an independent hybridisation event. Based on the analysis of expressed morphological characters it could be hypothesized that, in contrast to the European populations, *P. perfoliatus* constituted the maternal lineage here. Expression of morphological features, especially the apices of submerged leaves, presents a clear directional bias towards this parental species. Unfortunately, this hypothesis could not be tested at the molecular level, because no DNA was available from the African samples (see Materials and Methods). However, the occurrence of a pronounced matroclinal inheritance of morphological traits was also observed in other *P. nodosus* hybrids, *P. gramineus* × *P. nodosus* (Kaplan & Fehrer, unpub. data) and *P. ×subobtusus* (Zalewska-Gałosz & al., 2009), where different clones more closely resembled the female parent rather than having intermediate characters. Thus, the maternally driven expression of characters may account to a large degree for shaping the range of morphological variability in some *Potamogeton* hybrids.

An additional contribution to the large variation in character expression between Polish and African individuals of our hybrid taxon may be due to the involvement of different genotypes of the same parental species. Both *P. nodosus* and *P. perfoliatus* are regarded as variable (Wiegleb & Kaplan, 1998), showing a great amount of local morphotypes. Such large morphological variation is likely to reflect not only plant response to ecological conditions but also genetically inherited traits. However, it should be pointed out that differing habitat conditions could also largely drive the differences in quantitative characters. In

Poland *P. ×assidens* thrives in rivers (running water), while in Africa all hybrid plants were collected from stagnant or only slowly running water. It is well documented that habitat conditions considerably change the appearance of *Potamogeton* plants (Kaplan, 2002). In general, macrophytes growing in running water have a tendency to develop more elongated phenotypes, with longer internodes and submerged leaves, in comparison to phenotypes from stagnant water (Kaplan, 2008). Similar correlation is observed in the case of *P. ×assidens*.

Molecular assessment and probable matroclinal ITS homogenisation in hybrid plants. — High phenotypic plasticity of *Potamogeton* species causes numerous difficulties in morphological assessment of specimens and in setting precise morphological frames for particular taxa. Frequent interspecific hybridisation additionally complicates the diversity of forms present in nature. Some of the hybrid combinations are surprising in view of the great disparity of the characters in relation to the parents (Dandy, 1975). Often, it is extremely difficult to identify parental taxa of putative hybrid individuals presenting an altered morphology. In such cases, molecular characters may provide a decisive support for morphological and anatomical evaluation. The analysis of restriction profiles or sequences has been used to study and confirm several hybrid taxa previously described based on combinations of morphological and anatomical features (King & al., 2001; Kaplan & Fehrer, 2004, 2007; Zalewska-Gałosz & al., 2009). Finally, molecular techniques have also been recently applied in *Potamogeton* to support morphological identification and description of new taxa (Kaplan & al. 2009 and the present study). Although interpretation of ribosomal ITS sequences, especially in phylogenetic studies, can be misleading due to various molecular evolutionary mechanisms (e.g., Álvarez & Wendel, 2003), it proves to be a useful and efficient tool for analyses of recent hybrid formation in *Potamogeton* (e.g., Kaplan & Fehrer, 2007). In sexually reproducing hybrid generations, even though the extent and rate of concerted evolution is largely unpredictable (Álvarez & Wendel, 2003), it has been demonstrated that such mechanisms may drive the ITS sequences very quickly towards homogenization (Fuentes Aguilar & al., 1999). Agamic reproduction, however, can significantly retard concerted evolution (Campbell & al., 1997; Nieto Feliner & Rosselló, 2007). In the case of long-persisting but mostly sterile hybrid taxa of *Potamogeton*, the usual lack of crossing between F1 individuals and backcrossing probably slows down these mechanisms and in consequence conserves the biparental signal in the genome and facilitates interpretation of hybrid origin. In such cases, ITS lineages may be used as efficient markers of parental contribution to the hybrid genome (Soltis & al., 2008). In our study, the analysis of ITS variability provided a particularly clear and informative pattern of genetic variation. No intraspecific variation was observed in the parental taxa and all hybrid individuals displayed a rigorously additive pattern at sites polymorphic between *P. nodosus* and *P. perfoliatus*. Based on these very clear profiles, parental taxa could be identified with enough confidence without the need for separation of divergent ITS copies by cloning. All hybrids from both studied populations had identical sequences but a quantitative difference was observed in the ratio of nucleotide peaks from parental ITS lineages. In

the Drawa population the peaks were equally high while in the Rospuda populations the nucleotide variants from *P. nodosus* quantitatively dominated over those of *P. perfoliatus*. These differences might be due to occurrence of historical backcrosses in one population but it seems rather unlikely due to sterility observed among hybrid individuals. As mechanisms of concerted evolution act on the genomic ITS copies, this difference could be rather explained by different intensity of such molecular processes or by different age of the hybrid populations. Interestingly, the dominating ITS variant observed in the Rospuda individuals was that of *P. nodosus* which was identified based on cpDNA data as the maternal side of the hybrid. A significant matroclinal variation was also observed in the morphology of hybrids (see discussion above). Dominance of maternal genome expression is a feature well known for some plant groups, e.g., dog roses, but it is connected there with high ploidy levels and specific meiotic processes (e.g., Werlemark & Nybom, 2001). This is not the case in *Potamogeton*; examples of ITS homogenization in hybrid taxa towards the maternal lineage, however, have been reported for other plants (e.g., Brochmann & al., 1996) and also observed in another *Potamogeton* hybrid (Zalewska-Gałosz & al., 2009). Matroclinal inheritance of morphological features and genetic markers (AFLP phenotypes) was also observed, e.g., in a *Salix* hybrid complex (De Cock & al., 2003). It was demonstrated that ITS homogenization in hybrids can occur in different directions in various descendant lineages (Wendel & al., 1995) and represents a largely unpredictable process. Our observations of tendencies repeatedly found in *P. ×assidens* and several other *Potamogeton* hybrids may suggest a presence of genetic mechanisms promoting in some cases a higher impact of the maternal lineage on the expression and evolution of the hybrid variability.

Comparison of *P. ×assidens* and *P. ×rectifolius*, hybrids between *P. nodosus* and two closely related *Potamogeton* species. — As discussed above, the range of variability in expression of morphological characters by hybrids derived from the same parental species may be large and attributed, e.g., to matroclinal expression or contribution of various intraspecific genotypes. The case study of *P. ×assidens* provides a possibility to analyse the outcomes of hybridisation involving closely related parental species. It is essential to compare morphological characters between *P. ×assidens* and *P. ×rectifolius*. Both taxa are hybrids of *P. nodosus* differing by second parental species, which are *P. perfoliatus* and *P. richardsonii*, respectively. However, these are morphologically and phylogenetically very closely related species (Lindqvist & al., 2006; Kaplan & al., 2009). *Potamogeton ×rectifolius* was described from Illinois, U.S.A., as a hybrid between *P. americanus* (= *P. nodosus*) and *P. alpinus* (Bennett, 1902). However, according to a reappraisal of the material by Hagström (1916), this hybrid rather resulted from crossing between *P. nodosus* and *P. richardsonii*. This is supported by observations of Ogden (1943) and by the fact that the originally suggested parental species, *P. alpinus*, is unknown from Illinois (Haynes & Hellquist, 2000). For the same reason (absence in Illinois) *P. perfoliatus* is unlikely to be one of the parents (see also morphological differences described below). A lectotype for the name is selected from material formerly preserved in the personal herbarium of A. Bennett.

Potamogeton × *rectifolius* A. Benn. in J. Bot. 40: 147. 1902 (= *P. nodosus* × *P. richardsonii*) – Type: [original label:] Railroad ditches. “Stony Island”, Chicago. Ill., Aug. 18, 1900, coll. E. J. Hill. [annotation label of A. Bennett:] No. 8216, *P. americanus* × *alpinus* = × *Potamogeton rectifolius* Ar. Benn., Railway ditches. Stony Island. Chicago. Ill. United States. N. America., 18-8-[19]00, leg. Rev^d. E. J. Hill, com. Arthur Bennett, F.L.S., England (lectotype, designated here by Z. Kaplan: K; isolectotypes: BM, G, LE). Other syntypes (from the same site by the same collector but on different dates): Sept. 14, 1900 (BM, NY), Aug. 30, 1901 (BM, LD).

Potamogeton perfoliatus and *P. richardsonii* are morphologically very similar, as are their hybrids with *P. nodosus*. However, the hybrids differ in fine fibres present in decaying stipules at some nodes of *P. ×rectifolius* whereas stipules of *P. ×assidens* are translucent, delicate and disappearing completely with time. In general, the submerged leaves of *P. ×rectifolius* are narrower and their bases are not so clearly semiamplexicaul as those of *P. ×assidens*.

Comparison of *P. ×assidens* with other morphologically similar European hybrids. — Because of the generally higher diversity of known species in Europe compared to Africa, among the European hybrids there are several taxa displaying quite similar combinations of morphological features that could be confused with *P. ×assidens*. It concerns in particular some hybrids of *P. perfoliatus* and, surprisingly, of *P. alpinus*. These are: *P. ×nitens* (= *P. gramineus* × *P. perfoliatus*), *P. ×salicifolius* (= *P. lucens* × *P. perfoliatus*), *P. ×nericius* (= *P. alpinus* × *P. gramineus*), *P. ×nerviger* (= *P. alpinus* × *P. lucens*) and *P. ×subobtusus* (= *P. alpinus* × *P. nodosus*). Hybrids can be far more variable than pure species because of different and unpredictable parental gene combination and expression in a particular hybrid entity. As a consequence, indication of unambiguous and stable distinguishing characters between hybrids is sometimes difficult. However, some relatively consistent differences can be indicated, especially in characters of stipules, submerged leaves and in stem anatomy. *Potamogeton ×assidens* differs from *P. ×nitens* and *P. ×salicifolius* by producing fugacious stipules instead of persistent or subsistent ones as in the case of *P. ×nitens* and *P. ×salicifolius*. Also the leaf apex features differ; it is never mucronate in the case of *P. ×assidens*, while *P. ×nitens* and *P. ×salicifolius* often develop mucronate submerged leaves. The presence of semiamplexicaul leaf bases in *P. ×assidens* distinguishes it from *P. ×nericius* and *P. ×subobtusus*, which produce cuneate leaf bases, not even partly clasping the stem. Moreover, leaves of *P. ×subobtusus* are petiolate and much longer than those of *P. ×assidens*. In turn, *P. ×nericius* and *P. ×nerviger* develop cortical strands in the cortex, which are lacking in stems of *P. ×assidens*.

■ ACKNOWLEDGEMENTS

Our thanks are due to Adam T. Halamski, Jirka Danihelka, Ryszard Ochyra and Krzysztof Pawłowski for their help with Latin translation of the diagnosis; to Judith Fehrer, Wojciech Paul and two anonymous

reviewers for critical comments to the manuscript; and to the curators of the above-mentioned herbaria who allowed us to study their collections. J. Z.-G. was financially supported by grants no. 3PO4 G 089 23 and N303 098 32/3404 from the Polish Ministry of Science and Higher Education. The visit of J. Z.-G. to the collections and libraries of the Botanical Museum of the University of Copenhagen was supported by the European Commission's Integrated Infrastructure Initiative programme SYNTHESYS. Z. K. was supported by grant 206/09/0291 from the Grant Agency of the Czech Republic, and by the long-term institutional research plan no. AV0Z60050516 from the Academy of Sciences of the Czech Republic. The visits of Z. K. to the collections and libraries of the Botanical Museum of the University of Copenhagen, the Naturhistorisches Museum Wien, the Royal Botanic Garden Edinburgh and the Nationaal Herbarium Nederland in Leiden and Wageningen were supported by the European Commission's Integrated Infrastructure Initiative programme SYNTHESYS.

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Appendix 1. Features examined for morphological evaluation of *P. × assidens* individuals.

Stem: length, shape in cross-section, branching. *Submerged leaves: lamina*: length, width, length/width ratio, translucence/opacity, shape, colour, shape of base and apex, denticulation at margins, absence/presence and characteristics of lacunae, number of lateral veins, characteristics of secondary veins; *petiole*: absence/presence, length. *Transitional leaves: lamina*: length, width, length/width ratio, translucence/opacity, shape, colour, shape of base and apex, denticulation at margins, presence and characteristics of lacunae, number of lateral veins, characteristics of secondary veins; *petiole*: length. *Floating leaves: lamina*: length, width, length/width ratio, shape, colour, shape of base and apex, denticulation at margins, absence/presence and characteristics of lacunae, number of lateral veins, characteristics of secondary veins; *petiole*: length. *Stipules*: length, translucence/opacity, colour, persistence, characteristics of veins. *Inflorescence*: length, number and density of flower whorls. *Peduncle*: length, shape in cross-section.

Appendix 2. *Potamogeton* samples included in the DNA study.

Taxon; origin, date, collector; reference number; number of studied individuals; GenBank nos.: ITS; *rps12-rpl20*; *rpl32-trnL* (for part of samples).

P. × assidens Z. Kaplan, Zalewska-Galosz, M. Ronikier; NW Poland, Drawa River, 6 Jul. 2004, J. Zalewska-Galosz; 27/2004; 2; FJ883596; FJ883614; FJ883621. *P. × assidens* Z. Kaplan, Zalewska-Galosz, M. Ronikier; NE Poland, Rospuda River, 21 Jul. 2006, J. Zalewska-Galosz; 28/2004; 2; FJ883597; FJ883615; FJ883622. *P. alpinus* Balb.; SW Poland, Prószków, 16 Oct. 2006, A. Nowak; 81/2006; 1; FJ883580; FJ883598; FJ883616. *P. alpinus* Balb.; NW Poland, Krzywy Róg, 7 Jul. 2004, J. Zalewska-Galosz; 34/2004; 1; FJ883581; FJ883599. *P. alpinus* Balb.; S Poland, Dulowa, 5 May 2005, J. Zalewska-Galosz; 41/2004; 1; FJ883582; FJ883600. *P. nodosus* Poir.; S Poland, Wierchosławice, 13 Sept. 2006, M. Nobis; 73/2006; 1; FJ883593; FJ883611; FJ883620. *P. nodosus* Poir.; SE Poland, Ulanów, 27 Oct. 2006, A. Nobis; 76/2006; 1; FJ883594; FJ883612. *P. nodosus* Poir.; SW Poland, Kantorowice, 16 Oct. 2006, A. Nowak; 77/2006; 1; FJ883595; FJ883613. *P. lucens* L.; NE Poland, Rospuda River, 21 Jul. 2006, J. Zalewska-Galosz; 63/2006; 1; FJ883591; FJ883609; FJ883618. *P. lucens* L.; NW Poland, Drawa River, 6 Jul. 2004, J. Zalewska-Galosz; 35/2004; 2; FJ883592; FJ883610. *P. perfoliatus* L.; NW Poland, Kramsko Lake, 7 Aug. 2006, J. Zalewska-Galosz; 47/2006; 1; FJ883583; FJ883601; FJ883619. *P. perfoliatus* L.; SE Poland, Zwołaki, 27 Oct. 2006, A. Nobis; 75/2006; 1; FJ883584; FJ883602. *P. perfoliatus* L.; NW Poland, Sitno Lake, 9 Jul. 2004, J. Zalewska-Galosz; 36/2004; 1; FJ883585; FJ883603. *P. perfoliatus* L.; NW Poland, Drawa River, 6 Jul. 2004, J. Zalewska-Galosz; 37/2004; 1; FJ883586; FJ883604. *P. gramineus* L.; NW Poland, Chądzie Lake, 4 Aug. 2004, J. Zalewska-Galosz; 38/2004; 1; FJ883587; FJ883605; FJ883617. *P. gramineus* L.; E Poland, Kleszczów Lake, 4 Aug. 2004, J. Zalewska-Galosz; 40/2004; 1; FJ883588; FJ883606. *P. gramineus* L.; E Poland, Uściwierz Lake, 15 Aug. 2004, J. Zalewska-Galosz; 39/2004; 1; FJ883589; FJ883607. *P. crispus* L.; NW Poland, Olpuch-Wdyzde, 3 Aug. 2006, J. Zalewska-Galosz; 42/2006; 1; FJ883590; FJ883608.