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In search of speciation: Diversification of *Stuckenia pectinata* s.l. (Potamogetonaceae) in southern Siberia (Asian Russia)

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ABSTRACT

Although aquatic plants usually are less taxonomically diverse than related terrestrial groups, genetic studies could reveal groups of sibling species and thus increase taxonomic resolution. We hypothesised that different age and environmental diversity of water bodies in southern Siberia caused diversification within *Stuckenia pectinata* s.l. (Potamogetonaceae). To test this hypothesis we studied nuclear (ITS) and plastid (rpl32-trnL) genetic variation of 37 populations of *S. pectinata* from eastern Europe, the Russian Far East and southern Siberia with special emphasis on the latter. Plastid DNA variability within *S. pectinata* s.l. was found to be structured neither geographically nor taxonomically. We revealed strong ITS differentiation within *S. pectinata* s.l. in southern Siberian water bodies with different combinations of age and salinity. We discuss possible causes of such differentiation. We found a striking contrast between the absence of ITS variability across almost all of Europe colonized by the only ribotype, and relatively high ITS variability in southern Siberia. This contrast could be explained by the different history of young European populations that could have appeared as a result of recent northwards expansion following the Last Glacial Maximum, and the relatively old ones of southern Siberia. The absence of clear correspondence between genetic variation and morphological variability does not sufficiently support delimitation of species within Siberian *S. pectinata* s.l. (such as *S. chakassiensis* and *S. macrocarpa*).

1. Introduction

Aquatic plants (especially submerged ones) usually are less taxonomically diverse (in terms of number of recognized taxa) than related terrestrial groups (Barret et al., 1993; Santamaría, 2002). However, aquatics generally show a strong reduction in morphological traits and high intraspecific variation, which seriously constrain taxonomic clarity. On closer examination, however, particularly through the application of molecular tools, broadly distributed species might represent groups of sibling species (reviewed by Santamaría, 2002).

Genetic differentiation in a widespread and variable species *Stuckenia pectinata* (L.) Börner (Potamogetonaceae Dumort.) usually results from restricted gene flow (isolation by distance); the variable threshold distance above which populations showed restricted gene flow reaching up to 1000 km (Hollingsworth et al., 1996). This is due to propagule transport by waterfowl (Santamaría, 2002). Differentiation between populations over much shorter distances may result from adaptive responses to local differences which cause small-scale spatial segregation of ecotypes and further possible speciation (Santamaría, 2002). For example, a number of boreal species closely related to species with broad, boreal-temperate distributions (remnants of the Arcto-Tertiary flora) were suggested to evolve due to ecological differentiation of sympatric species in their northern range. One of well-known examples of such diversification could be found in the genus *Stuckenia* Börner, which includes subcosmopolitan *S. pectinata* and boreal *S. filiformis* (Pers.) Börner and *S. vaginata* (Turcz.) Holub (Santamaría, 2002).

Further taxonomic differentiation within *Stuckenia pectinata* could be expected as, in spite of effective propagule transport, within-species isolation by distance was revealed not only on area-wide scale (Mader et al., 1998), but also on local level, e.g. in the Baltic Sea basin (King et al., 2002; Nies and Reusch, 2005). More pronounced isolation by distance was revealed between lakes due to their lower connectivity (Nies and Reusch, 2005). Even in continuous marine habitat some genetic differentiation was found to exist due to environmental heterogenity (King et al., 2002; Nies and Reusch, 2005). However, the strongest genetic differentiation, suggesting almost complete reproductive isolation, was observed between freshwater and marine populations situated close to each other. This may indicate that local adaptation to contrasting environmental conditions, e.g. salinity (Nies

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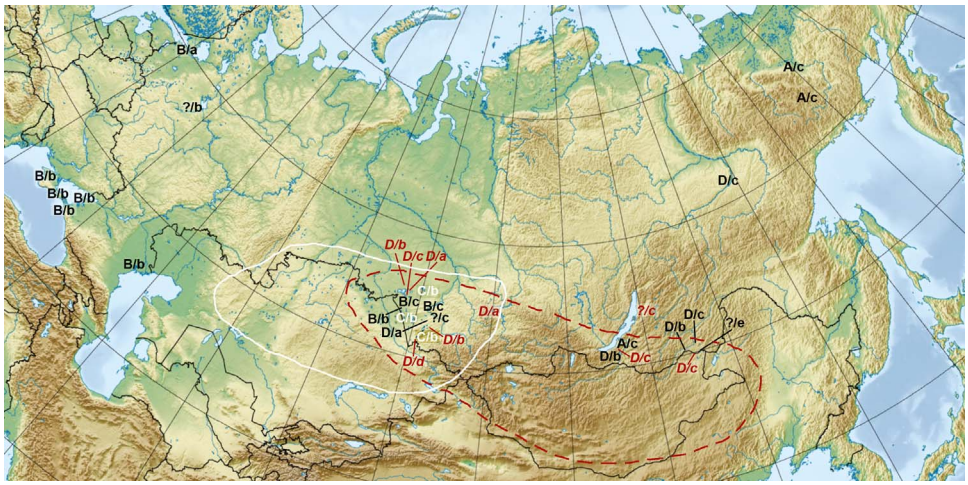

Fig. 1. Geographical distribution of *Stuckenia pectinata* s.l. genetic variability: ITS ribotypes/rpl32-trnL haplotypes are shown (in upper and lower case letters respectively, cf. Figs. 2 and 4, Table 2). Each labeling indicates one–four populations. ITS ribotypes with polymorphic positions are marked as "?". Plants, tentatively referred to *S. macrocarpa*, are indicated by white colored letters and the range of this taxon (according to Lisitsyna, Papchenkov, 2000; Kaplan, 2008; original data) is outlined by the solid white line. Plants tentatively referred to *S. chakassiensis* are indicated with red colored, italicized letters and the range of this taxon (according to Kashina, 1986; Volobaev, 1991, 1993; original data) is outlined by red dashed line. Plants tentatively referred to *S. pectinata* s.str. are indicated with black colored letters; the range of this subcosmopolitan taxon is not shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and Reusch, 2005), is important for shaping the genetic diversity of *S. pectinata* (Triest et al., 2010). The observed differentiation is interpreted as an example of rapid (post-glacial) incipient speciation in a widespread aquatic plant (Nies and Reusch, 2005).

Although genetic variation of *Stuckenia pectinata* is well-studied in Europe, especially in its western part (reviewed by Triest et al., 2010), the vast south Siberian part of the species range that represents the centre of *Stuckenia* diversity (Kaplan, 2008) remains unexplored. Southern Siberia is characterized by a complex relief and high diversity of water body types, especially lakes, varying in their mineralization and history (Kipriyanova, 2007; Durnikin, 2013; Chepinoga, 2015). Relic (pre-Quaternary) Siberian water systems could serve as refugia where genetic diversity could accumulate for a long time. This leads us to expect some genetic differentiation within Siberian *S. pectinata* s.l.

There are numerous evaluations of the extensive morphological variation of *Stuckenia pectinata* in the taxonomic literature. It is possible that such a variable species consists of several distinct evolutionary lineages, although this remains to be tested with molecular data (Kaplan, 2008). In particular, two species were described as abundant in southern Siberia (Fig. 1): *S. macrocarpa* (Dobrochot.) Tzvelev (Lisitsyna and Papchenkov, 2000; Kaplan, 2008) and less confidently, *S. chakassiensis* (Kaschina) Klinkova (Volobaev, 1991, 1993; Tzvelev, 1999). The former could be most reliably distinguished from *S. pectinata* s.str. by larger fruits and the latter is characterized by presence of subepidermal sclerenchymatous strands in leaves. Other characters, proposed as diagnostic (e.g. length of the leaves and ligula), are not believed to be reliable (Kaplan, 2008; Table 1). Such subtle differences and the high ecological plasticity of the taxa led some authors to refrain from delimiting either *S. chakassiensis* (e.g. Kaplan, 2008) or *S. macrocarpa* (e.g. Sviridenko, 2000).

We hypothesised that the different age and environmental diversity of water bodies in southern Siberia has caused diversification within *Stuckenia pectinata*. To test this hypothesis we studied nuclear (ITS) and plastid (rplL32-trnL) genetic variation *S. pectinata* s.l. in this region. We expected that any divergent selection will eventually be reflected at the analyzed neutral genetic markers as well, even if the latter were not the target of selection (Nies and Reusch, 2005). We also aimed to test whether the observed genetic variation corresponded with morphological variability, thus supporting delimitation of any taxa within *S. pectinata* s.l.

2. Materials and methods

2.1. Sampling

In 2003–2015 we collected 37 samples of *Stuckenia pectinata* s.l. (including plants, tentatively referred to *S. chakassiensis* and *S. macrocarpa*), from eastern Europe, the Russian Far East and southern Siberia with special emphasis on the latter (Table 1, Fig. 1). We also included one–two samples of other representatives of the genus occurring in Siberia (*S. filiformis*, *S. vaginata* and *S. subretusa* (Hagstr.) Holub (sometimes treated as a form of *S. vaginata*: Kaplan, 2008)) that were used as an outgroup in accordance to the recent phylogeny of Potamogetonaceae (Lindqvist et al., 2006). Taxa identification was performed in conformance with published diagnostic characters (Table 1). Leaf samples for DNA extraction were dried in most cases in silica gel (rarely were taken from herbarium samples). Five–ten plants per population were pressed as vouchers (preserved in herbarium of Novosibirsk branch of Institute for water and environmental problems SB RAS and in IBIW, Russia). Water mineralization was measured directly in the field using compact multiparameter analytical testers ANION-7051 and Hanna HI 98129. We extracted approximate age of the reservoirs from the relevant literature (Florensov et al., 1968; Svitoch and Yanina, 1994; Kipriyanova, 2007; Geniatulin, 2009; Durnikin, 2013; Chepinoga, 2015).

2.2. DNA extraction, PCR amplification and sequencing

We analyzed one plant per population as no within-population genetic variability on the selected markers was detected in the preliminary study. Between 10–15 mg of dried plant material was used for DNA isolation. The sample plant tissue was ground to a fine powder using Mixer Mill 400 (Retsch) and 3-mm tungsten beads. The total genomic DNA was extracted using a K-Sorb Column Kit (Syntol), following the manufacturer's protocol (final elution step used 2 × 50 µl elution buffer). Quality of DNA extractions was roughly verified by Nanophotometer P300 (Implen). The nuclear ribosomal internal transcribed spacer region (including ITS1, 5.8S and ITS2) was amplified using universal primers ITS1A (forward) 5′-TCGTAACAAGGTTTCCGTAGGTG-3′ and ITS4 (reverse) 5′-TCCTCCGCTTATTATTGATATGC-3′ (White et al., 1990; Fuertes Aguilar et al., 1999). Although all the *Stuckenia* taxa are hexaploids (2n = 78: Kaplan et al., 2013) which could cause existence of multiple homologous copies of a gene, ITS remains a reliable region for the genus systematics (Kaplan et al., 2013; Yang et al., 2016). The following reaction composition was applied in a total volume of 25 µl: 5 µM ScreenMix-HS (Evrogen), 0.5 µM of each primer, 18 µM H_2O 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 decrease of 0.4 °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.

The chloroplast intergenic spacer rpl32-trnL was amplified using universal primers rpl32 (forward) 5′-CAGTTCCAAAAAAACGTACTTC-3′

Table 1
Diagnostic characters (Wiegleb and Kaplan, 1998; Bobrov, 2007; Kaplan, 2008; original data) and water mineralization preferences (Kipriyanova et al., 2007, 2016, 2017; original data) of *Stuckenia* taxa in Siberia.

Characters	*S. pectinata*	*S. chakassiensis*	*S. macrocarpa*	*S. filiformis*	*S. vaginata*	*S. subretusa*
Length of stem leaves, cm	3–12(20)	4–12(15)	2–6(8)	3–18(24)	2–10(13)	2–15(20)
Leaf apex	acute to acuminate, mucronate	acute to acuminate, mucronate	acuminate, mucronate	obtuse to acute	truncate to acute, subretuse	truncate, subretuse
Subepidermal sclerenchymatous strands in leaves	absent	present	absent	absent	absent	absent
Presence of short leaves in the lower part of the stem	occur	present	occur	absent	present	present
Branching pattern	mostly richly branched mainly above	moderatly branched to middle	mostly densely branched above, particularly on terminal parts	mostly richly branched near base and unbranched above	richly branched mainly above	sparingly branched to middle
Number of branches arising from the node in the middle part of the stem	2	2	2	2	2–5	2–3
Leaf sheaths	open	open	open	tubular	open	open
Length of sheaths, cm	1–7	1–5	1–3	0.5–3(5)	2–8	2–7
Width of sheaths, mm	0.5–4.2	0.5–5	0.5–2	0.5–2	2–10	2–6
Length of ligula, mm	5–15	1–15	< 1	5–15	0.5–4	4–10
Stigmas	stalked	stalked	stalked	sessile	sessile	sessile
Length of fruits, mm	3.3–4.2(4.5)	3.5–4.7(5.1)	(4.1)4.3–5.8	2–2.8(3.2)	2.5–3.8(4)	2.2–2.8(3)
Range of water mineralization, g/dm^3	0.1–6.5	1–28.8	0.3–4.5	0.1–1.5	0.1–2.6	< 0.2

and trnL (reverse) 5′-CTGCTTCCTAAGAGCAGCGT-3′ (Shaw et al., 2007). This region has been shown to be the most variable in our preliminary study (L. Kipriyanova, unpubl.), revealing at least some variability within *S. pectinata* s.l. (see Section 3.2). The same reaction mix composition as described above was used. 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 T100 thermal cycler (Bio-Rad).

PCR products were purified and sequenced in both directions employing the primers used for amplification. Sequencing was performed using BigDye Terminator ver. 3.1 (Applied Biosystems) with supplied 5 × sequencing buffer, according to the manufacturer's manual. Samples were purified using the Ethanol/EDTA procedure, resuspended in 10 µl formamide and separated on an ABI 3100 Genetic Analyzer using 50 cm capillaries and POP-6 polymer (Applied Biosystems).

2.3. Molecular data analyses

Raw sequencing profiles were analyzed using the DNA Sequencing Analysis Software version 5.1 (Applied Biosystems). The sequences were manually verified/adjusted using Sequencher 4.1.4 software (Gene Codes Corp.). Alignments of sequences of all regions were conducted manually using BIOEDIT 7.2.5. software (Hall, 1999). Additive nucleotide polymorphisms were examined with two strands to ensure their consistency and coded using IUPAC nucleotide ambiguity codes (these sequences were not included into parsimony and maximum likelihood analyses). All sequences were deposited to GenBank (alignments ITS, rpl32-trnL: accession no. KY407929—KY407969, KY407970—KY408010).

We carried out statistical parsimony analysis using the network algorithm described in Templeton et al. (1992) and implemented in the TCS v. 1.21 program (Clement et al., 2000). This method estimates the unrooted haplotype network and a 95% plausible set of all haplotype lineages in that network (gaps were treated as missing data).

Maximum likelihood (ML) analysis and tree building was performed in R (R Development Core Team, 2009), using packages Ape and Phangorn (gaps were treated as missing data). Bootstrap values were estimated with 1000 bootstrap samples.

3. Results

3.1. ITS sequences

The alignment was 628 positions long. Within the outgroup *Stuckenia subretusa* differed from *S. filiformis* in two positions and from *S. vaginata* in an additional position (shared with *S. filiformis*). The TCS program calculated the 95% limit of parsimony of two mutational steps, that was insufficient to connect all the ribotypes of *S. pectinata* s.l. (differing in 10 polymorphic positions) in a single network (not shown). All the four ribotypes of the ingroup were united in a single network only when the connection limit was manually increased to seven mutation steps (Fig. 2). The outgroup was still too genetically distant to be included in the network (not shown). To root the network, we performed ML analyses on samples of *S. pectinata* s.l. and the outgroup (Fig. 2). The most likely substitution model was JC, the tree for this was 32 steps long (consistency and retention indices were equal to 1).

The ML analysis connected the outgroup with the ribotype **A** found in the three *Stuckenia pectinata* s.str. samples from young (< 1000 years old), eastern fresh water bodies (Figs. 1 and 3, Table 2). This ribotype was separated by seven mutations from the nearest ribotype **B**, found in *S. pectinata* s.str. from relatively young (< 7000 years old), western brackish water bodies. Close ribotype **C**, differing from **B** in two nucleotide positions, was revealed in *S. macrocarpa* from young fresh water bodies of south-western Siberia. The south Siberian ribotype **D** was found to be most distant from the outgroup, differing from ribotype **C** by one substitution. It was found in *S. chakassiensis* from old (> 10 000 years old) brackish lakes and in *S. pectinata* s.str. from young mainly fresh (with one exception) lakes. The association between age class of water body (young vs. old) and combination of ribotype and taxa is highly significant (Chi-square test: $p = 0.0005$).

ITS sequences of four plants, determined as *Stuckenia chakassiensis* and *S. pectinata* s.str., from different parts of the study area included several (1–9) polymorphic sites (additive nucleotide polymorphisms).

3.2. cpDNA sequences

The alignment was 694 positions long. Within the outgroup *Stuckenia subretusa* and *S. vaginata* were closer to each other, differing in one position. *Stuckenia filiformis* deviated from these two species on seven nucleotide positions and five indels that were 1–9 positions long.

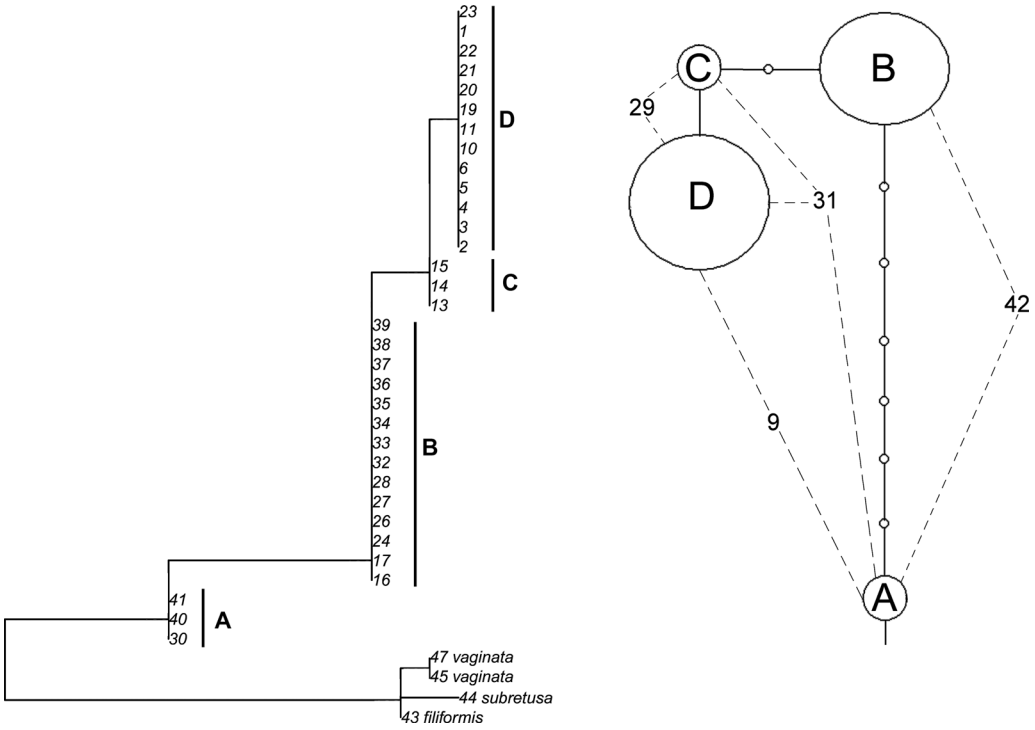

Fig. 2. The most parsimonious tree (left) and statistical parsimony network (right) of *Stuckenia pectinata* s.l. ITS ribotypes. The ITS sequences with polymorphic sites (populations 9, 29, 31, 42: Table 2) were excluded from analyses and were manually added to the network, connecting possible variants of the polymorphic sequences (dotted lines). Other Siberian representatives of *Stuckenia* (*S. filiformis*, *S. subretusa* and *S. vaginata*) were included in the tree as an outgroup (according to Lindqvist et al., 2006). All bootstrap values were equal to 100 and therefore are not indicated on the tree. The sizes of network nodes are proportional to the number of populations representing each ribotype. Lines on the network represent the mutational pathway interconnecting the ribotypes; dots represent inferred intermediate ribotypes which were not observed in the data.

The TCS program calculated the 95% limit of parsimony of two mutational steps, revealing five haplotypes within *Stuckenia pectinata* s.l. (there were four polymorphic positions). The outgroup was too distant genetically to be included in the network (not shown). To root the network, we performed ML analyses on samples of *S. pectinata* s.l. and the outgroup (Fig. 4). The most likely substitution model was GTR; the tree for this was 34 steps long (consistency and retention indices were equal to 1).

The ML analysis connected the outgroup with the most widespread haplotype **b**, which was found in all the three taxa within *Stuckenia pectinata* s.l. across all of the study area with the exception of its north-eastern margin (Figs. 1 and 4). The haplotype **a** differed from **b** by one substitution and was found in two populations of *S. pectinata* s.str. from the Gulf of Finland and in a fresh-water lake in south-western Siberia, and in two populations of *S. chakassiensis* from the latter region (Figs. 1 and 4). The haplotype **c** also differed from haplotype **b** by one nucleotide and was found in populations of Siberian *S. pectinata* s.str. and *S. chakassiensis*. Two rare haplotypes **d** and **e** differed from **c** in one position. They were found in one population of *S. chakassiensis* from south-western Siberia and one population of *S. pectinata* s.str. from south-eastern Siberia respectively.

4. Discussion

4.1. Comparison of phylogenies, based on plastid and nuclear markers

Our studies demonstrate that the distribution of the haplotypes of maternally inherited cpDNA and biparentally inherited ITS within *Stuckenia pectinata* s.l. is not congruent, as was shown already for the genus (Yang et al., 2016). In spite of possible homoplasy, ITS is well-suited for species delimitation within Potamogetonaceae (Kaplan et al., 2013; Yang et al., 2016). In contrast, cpDNA haplotypes are usually shared among closely related taxa, which is explained by chloroplast capture due to hybridization which is widespread in *Stuckenia* (e.g.

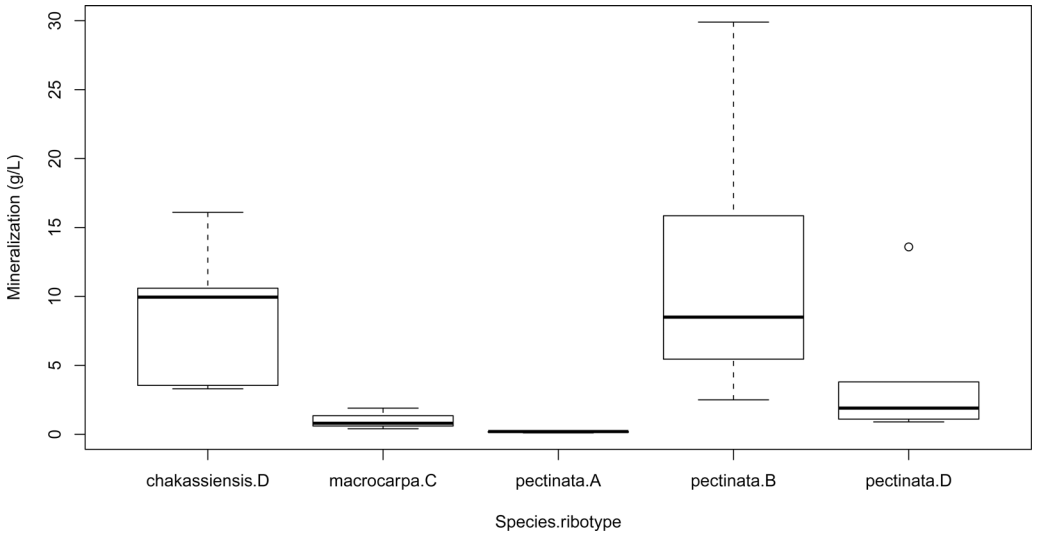

Fig. 3. Mineralization of water where plants with different ribotypes tentatively referred to different taxa within *Stuckenia pectinata* s.l. were collected (cf. Fig. 2, Table 2). Median; absolute and quartile ranges are represented.

Table 2
Geographic origin, mineralization and approximate age of water bodies, ITS ribotype and plastid haplotype (cf. Figs. 2 and 4) of the investigated *Stuckenia* samples. Tentative determinations based on morphology were made by L.M. Kipriyanova, A.A. Bobrov.

No.	Taxon	ITS ribotype[a]	*rpl32-trn*L haplotype	Origin	Coordinates: latitude, N longitude, E	Mineralization, g/dm^3	Age of water bodies, thousand years[b]
1	*S. chakassiensis*	D	a	Novosibirsk reg., Barabinsk distr., 6 km to SW from village Kazantsevo, lake Chany, 26.07.2013, coll. LK	55°03′13” 77°38′40”	10.3	> 10
2	*S. chakassiensis*	D	c	Novosibirsk reg., Barabinsk distr., 1 km to W from village Novoyarkovo, lake Chany, Yarkovskii pool, 19.06.2014, coll. LK	54°54′02” 78°03′18”	10.3	> 10
3	*S. chakassiensis*	D	b	Novosibirsk reg., Chany distr., 4.5 km to NE from village Chany, lake Embakul, 25.07.2015, coll. LK	55°20′50” 76°50′49”	3.3	> 10
4	*S. chakassiensis*	D	d	Altai terr., Uglovskoe distr., 1.3 km to NW from village Krugloe, lake Krugloe, 14.07.2014, coll. LK	51°17′42” 80°25′34”	3.6	> 10
5	*S. chakassiensis*	D	b	Altai terr., Romanovo distr., near village Mamontovo, lake Maloe Gorkoe, 09.07.2014, coll. LK	52°39′25” 81°30′56”	16.1	> 10
6	*S. chakassiensis*	D	a	Rep. of Khakassia, Shira distr., village Zhemchuzhnyi, lake Shira, 19.09.2014, coll. LK	54°29′14” 90°10′57”	10.9	> 10
9	*S. chakassiensis*	?	c	Rep. of Buryatia, Barguzin distr., 60 km NE from village Barguzin, north part of lake Nukhe-Nur, 06.08.2014, coll. LK	54°01′51” 110°16′60”	2.0	> 10
10	*S. chakassiensis*	D	c	Rep. of Buryatia, Selenga distr., 9 km NNE from town Gusinoozyorsk, lake Sulfatnoe, 11.08.2014, coll. LK	51°21′43” 106°34′31”	9.6	> 10
11	*S. chakassiensis*	D	c	Chita reg., Ononskii distr., 6 km to N from village Narym-Bulak, lake Narym-Bulak, 22.07.2014, coll. LK	50°19′05” 115°19′08”	3.5	> 10
13	*S. macrocarpa*	C	b	Altai terr., Volchikha distr., village Seliverstovo, lake Ubiennoe, 10.07.2014, coll. LK	52°18′07” 80°57′51”	0.4	< 1
14	*S. macrocarpa*	C	b	Altai terr., Burla distr., 4.6 km to NE from village Novoalekseevka, lake Peschanoe, 17.07.2013, coll. LK	53°24′26” 78°38′16”	1.9	< 1
15	*S. macrocarpa*	C	b	Novosibirsk reg., Kargat distr., 3.3 km to NE from town Kargat, river Kargat, 21.07.2015, coll. LK	55°12′59” 80°20′08”	0.8	< 1
16	*S. pectinata*	B	b	Rep. of Crimea, Kirovskoe distr., village Vladislavovka, pond, 13.08.2015, coll. LK	45°09′35” 35°22′28”	8.5	< 1
17	*S. pectinata*	B	b	Rep. of Crimea, Krasnoperekopsk distr., 4.2 km to NNW from village Novoaleksandrovka, lake Aigulskoe, pond, 15.08.2015, coll. LK	45°54′43” 34°04′07”	8.7	< 1
19	*S. pectinata*	D	a	Altai terr., Zaviyalovo distr., 8 km to NE from village Kharitonovo, lake Mostovoe, 07.07.2014, coll. LK	53°04′43” 80°49′58”	0.9	< 1
20	*S. pectinata*	D	b	Rep. of Buryatia, Dzhidinskii distr., 13.9 km to SSW from village Borgoi, unnamed small pool close to lake Verkhnee Beloe, 12.08.2014, coll. LK	50°37′35” 105°45′49”	1.9	< 1
21	*S. pectinata*	D	b	Chita reg., Ononskii distr., 12.4 km SE from village Builesan, unnamed lake near lake Tsagan-Nur, 26.07.2014, coll. LK	50°10′39” 114°59′58”	3.8	< 1
22	*S. pectinata*	D	c	Chita reg., Ononskii distr., lake near village Novaya Zarya, 22.07.2014, coll. LK	50°20′56” 115°33′54”	13.6	< 1
23	*S. pectinata*	D	c	Rep. of Sakha (Yakutia), Khangalasskii distr., near village Mokhsogollokh, 91 km of Pokrovskii tract, alas lake to the left of the road, 22.07.2014, coll. E.V. Chemeris, E.G. Nikolin	61°25′12” 128°55′36”	1.1	< 1
24	*S. pectinata*	B	c	Altai terr., Baevo distr., 5 km to W from village Baevo, lake Lena, 06.07.2014, coll. LK	53°16′09” 80°41′29”	4.2	< 1
26	*S. pectinata*	B	c	Novosibirsk reg., Krasnozerskoe distr., 4 km to SW from village Konevo, lake Konevo, 12.08.2003, coll. LK	54°14′21” 79°21′51”	2.7	< 1
27	*S. pectinata*	B	b	Novosibirsk reg., Karasuk distr., 3.7 km to SSW from village Blagodatnoe, lake Krivoe, stretch Sopatoe, 21.07.2013, coll. LK	53°48′33” 78°01′56”	2.5	< 1
28	*S. pectinata*	B	c	Novosibirsk reg., Karasuk distr., 2 km to SE from village Astrodym, lake Astrodym, 22.07.2013, coll. LK	53°38′04” 77°47′55”	6.7	< 1
29	*S. pectinata*	?	c	Altai terr., Baevo distr., 8 km to W from village Baevo, lake Travnoe, 06.07.2014, coll. LK	53°15′04” 80°38′35”	3.1	< 1
30	*S. pectinata*	A	c	Rep. of Buryatia, Seleginsk distr., 7.7 km to SW from village Zharganta, lake Kamyshinoe, 15.08.2014, coll. LK	51°23′29” 106°33′25”	0.2	< 1
31	*S. pectinata*	?	e	Chita reg., Ononskii distr., 0.4 km to W from village Kulusutai, pool of lake system Barun-Torei, 25.07.2014, coll. LK	50°14′06” 115°40′05”	0.6	< 1
32	*S. pectinata*	B	a	Leningrad reg., Vyborg distr., opposite of town Ozerki, Gulf of Finland, sandy beach, 21.07.2013, coll. E.A. Movergoz	60°11′44” 29°00′23”	–	< 4
33	*S. pectinata*	B	b	Astrakhan reg., Narimanov distr., near village Prikaspiiskii, ilmen, 21.08.2010, coll. V.G. Papchenkov, A.P. Laktionov	46°13′24” 47°11′12”	–	< 5
34	*S. pectinata*	B	b	Astrakhan reg., Narimanov distr., near village Yango-Asker, ilmen Baldy-Kashkai, 20.08.2010, coll. V.G. Papchenkov, A.P. Laktionov	46°15′21” 47°38′35”	–	< 5
35	*S. pectinata*	B	b	Krasnodar reg., Novorossiisk bay, lagoon Sudzhukskaya, 26.08.2015, coll. LK	44°41′05” 37°48′04”	14.3	< 7
36	*S. pectinata*	B	b	Rep. of Crimea, Lenino distr., village Priozyornoe, lake Churbashskoe, 09.08.2015, coll. LK	45°16′04” 36°20′45”	8.2	< 1
37	*S. pectinata*	B	b	Rep. of Crimea, Lenino distr., 3.7 km to E from village Zavodskoe, pond in the system lake Aktashskoe, 10.08.2015, coll. LK	45°21′12” 35°46′44”	29.9	< 1
38	*S. pectinata*	B	b	Rep. of Crimea, Sevastopol, bay Streletskaya, 18.08.2015, coll.	44°35′37”	17.5	< 7

(continued on next page)

Table 2 *(continued)*

No.	Taxon	ITS ribotype[a]	*rpl32-trn*L haplotype	Origin	Coordinates: latitude, N longitude, E	Mineralization, g/dm^3	Age of water bodies, thousand years[b]
				LK	33°28′14”		
39	*S. pectinata*	B	b	Krasnodar reg., Eisk distr., village Dolzhanskaya, salt pool near sea of Azov, 28.08.2015, coll. LK	46°40′32” 37°44′37”	17.4	< 1
40	*S. pectinata*	A	c	Magadan reg., Susuman distr., 17 km to SE from village Burkandiya, interfluve of rivers Byoryolyokh and Malyk-Sien, lake Okunyovoe, 24.08.2012, coll. AB, O.A. Mochalova	63°17′29” 147°50′15”	0.2	< 1
41	*S. pectinata*	A	c	Rep. of Sakha (Yakutia), Verkhnekolymskii distr., 5 km to SW from village Nelemnoye, left bank of river Yasachnaya, river Mamonta, lower part, 24.08.2014, coll. AB, O.A. Mochalova	65°27′18” 151°01′05”	0.1	< 1
42	*S. pectinata*	?	b	Yaroslavl reg., Nekouz distr., Rybinsk reservoir, bay on river Ild, 07.08.2013, coll. E.A. Movergoz	58°01′11” 38°15′16”	–	< 1
43	*S. filiformis*	–	–	Rep. of Sakha (Yakutia), Mirnyi distr., village Morkoka, river Morkoka, 23.07.2015, coll. AB et al.	63°36′17” 112°30′49”	0.05	< 1
44	*S. subretusa*	–	–	Rep. of Sakha (Yakutia), Verkhnekolymskii distr., 5 km to SW from village Nelemnoye, left bank of river Yasachnaya, river Mamonta, lower part, 24.08.2014, coll. AB, O.A. Mochalova	65°27′18” 151°01′05”	0.1	< 1
45	*S. vaginata*	–	–	Rep. of Buryatia, Selenginsk distr., 12 km to NNE from town Gusinoozyorsk, lake Krugloe, 15.08.2014, coll. LK	51°23′49” 106°31′34”	0.1	> 10
47	*S. vaginata*	–	–	Chita reg., Aginskii distr., 13 km to N from village Budulak, system of lakes Khulusun, small unnamed lake, 31.07.2014, coll. LK	50°40′24” 114°54′01”	1.0	> 10

Names of some collectors are abbreviated: LK — Laura M. Kipriyanova, AB — Alexander A. Bobrov.

[a] ?ITS sequences with polymorphic sites that could not be attributed to one of the revealed ribotypes.

[b] Age of water bodies (Florensov et al., 1968; Svitoch and Yanina, 1994; Kipriyanova, 2007; Geniatulin, 2009; Durnikin, 2013; Chepinoga, 2015).

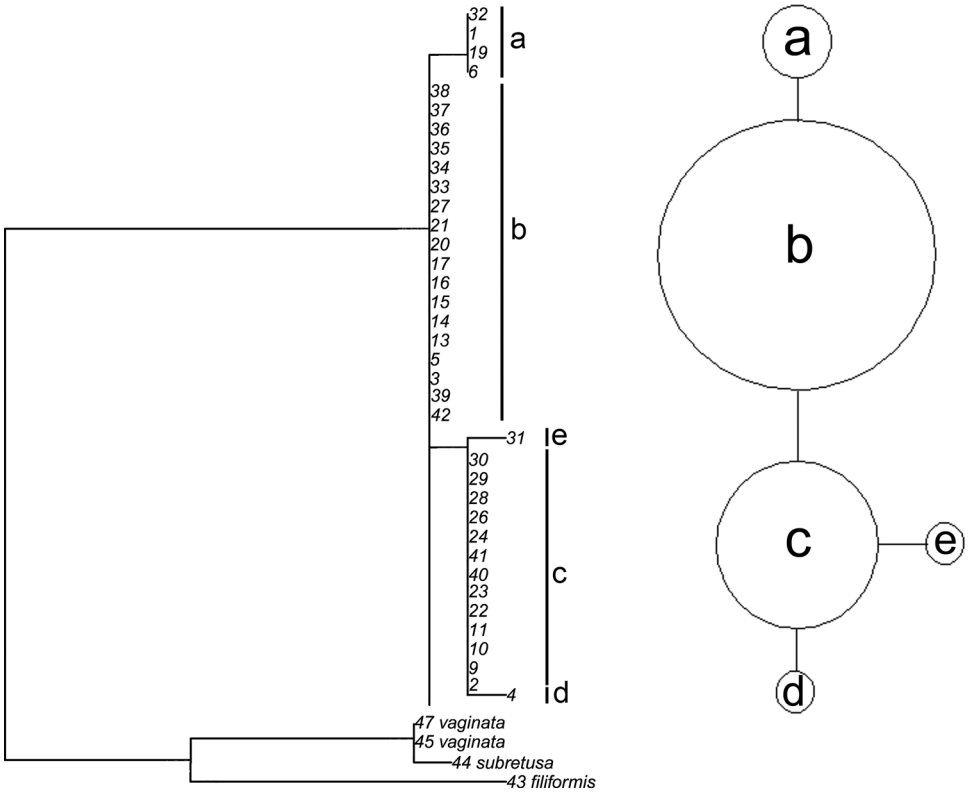

Fig. 4. The most parsimonious tree (left) and statistical parsimony network (right) of *Stuckenia pectinata* s.l. rpl32-trnL plastid haplotypes. Other Siberian representatives of Stuckenia (*S. filiformis, S. subretusa* and *S. vaginata*) were included in the tree as an outgroup (according to Lindqvist, et al., 2006). All bootstrap values were equal to 100 and therefore are not indicated on the tree. The sizes of network nodes are proportional to the number of populations representing each haplotype. Lines on the network represent the mutational pathway interconnecting the haplotypes; dots represent inferred intermediate haplotypes which were not observed in the data.

McMullan et al., 2011; Kaplan et al., 2013), and/or by incomplete lineage sorting, when common haplotypes and their derivatives are inherited from the nearest common ancestor (Schmidt-Lebuhn et al., 2012). Chloroplast capture resulting from hybridization was also described in the closely related genus *Ruppia* L. (Triest and Sierens, 2014). Although interspecific hybrids in *Stuckenia* are fully sterile (Preston et al., 1998; Preston et al., 1999; Bobrov, 2007), blocking chloroplast capture between them, hybrids between *S. pectinata* s.l. with different haplotypes should be at least partly fertile (see Section 4.2). Recent hybridization within *S. pectinata* s.l. is suggested by the revealed polymorphisms in the ITS sequences of four samples. Plastid DNA variability within *S. pectinata* s.l. is clearly structured neither geographically nor taxonomically (Fig. 1). Accordingly, we base further taxonomic and biogeographic inferences on the ITS and not plastid DNA variation.

4.2. Diversification within Stuckenia pectinata s.l

We found strong genetic diversification within Eurasian *Stuckenia pectinata* s.l., that has been already reported in Europe, where the ribotype **A** was found in the northeastern Europe (Pechora delta) and the ribotype **B** was revealed across western Europe: Scotland, the Netherlands, northern Italy (McMullan et al., 2011), German Baltic coast (Prof. T. Reusch, pers. comm. on 18.12.2016; Nies and Reusch, 2005) and Switzerland (Kaplan et al., 2009). Both ribotypes were also found in China (Wang et al., 2007; Yang et al., 2016) and north-eastern USA (Genbank accession numbers EF526376, EF526376; not published), ribotype **B** was additionally reported from India (Kaplan et al., 2013). The south Siberian ribotypes **C** and **D** to our best knowledge have not been described previous to the present study.

Our data do not sufficiently support specific delimitation of *Stuckenia chakassiensis* and *S. macrocarpa*. Numerous attempts to evaluate extensive morphological variation of *Potamogeton* L. and *Stuckenia* taxonomically were based on highly plastic characters (Kaplan, 2002, 2008). The case of *S. chakassiensis* exemplifies this, as the degree of development of subepidermal sclerenchymatous strands in leaves (its main diagnostic character) is related to the degree of water salinity (Kipriyanova, 2007), and we found plants tentatively referred to as *S. chakassiensis* only in brackish waters (Fig. 3). Moreover, plants referred to as *S. chakassiensis* could not been genetically distinguished from some freshwater plants of *S. pectinata* s.str. sharing the same ribotype **D**.

Species status for *Stuckenia macrocarpa* is more credible since its main diagnostic character (size of fruits) has been proven to be stable over large geographical areas and a wide range of environmental conditions (Kaplan, 2008). In our study plants referred to as this taxon are characterized by private ribotype **C**. Their genetic differentiation, however, falls within the limit of genetic variability of *S. pectinata* s.str. Thus in the absence of firm evidence, we do not consider *S. macrocarpa* to represent a distinct species.

Moreover, we detected four specimens with additive polymorphisms in ITS sequences (Table 1, Fig. 2) which should represent recent hybrids between *S. pectinata* s.l. with different ribotypes (Kaplan et al., 2009). The hybrid plants were revealed in areas where populations with parental ribotypes occur together or nearby. It is well-known that interspecific hybrids in *Stuckenia* are fully sterile, do not produce fruits and have misshapen, not filled pollen. This has been demonstrated convincingly for *S.* × *bottnica* (Hagstr.) Holub. (*S. pectinata* × *S. vaginata*) (Preston et al., 1998), *S.* × *fennica* (Hagstr.) Holub. (*S. filiformis* × *S. vaginata*) (Bobrov, 2007) and *S.* × *suecica* (K. Richt.) Holub. (*S. filiformis* × *S. pectinata*) (Preston et al., 1999). The same could be expected if plants with different ribotypes belong to different species within *S. pectinata* s.l. But this was not the case as all plants with additive patterns in ITS did not show any traces of sterility, they have well-developed fruits or visible viable carpels and fertile well-formed, filled pollen.

The detected distribution of the most ancient ribotype **A** (mainly in North America and eastern Asia) agrees with the proposed early Tertiary origin of the genus in these regions, connected at that time with North Atlantic land bridge (Lindqvist et al., 2006). In such a circumstance *Stuckenia pectinata*, bearing the most widely distributed ribotype **B**, could then have spread across all Eurasia (probably aided by waterfowl migrating mainly in a latitudinal direction: Mader et al., 1998) and could also have experienced further diversification (ribotypes **C** and **D**) in southern Siberia. The numerous extinct steps of evolutionary divergence evident between the ribotypes **A** and **B** (shown by gaps on the network) suggest local extinctions and limited dispersal of the ancient populations, during their possible expansion across Eurasia. A young South-to-North dispersal from the continental regions should also not be excluded, but wider sampling is needed to test this hypothesis.

The revealed ITS variability (10 substitutions) within *Stuckenia pectinata* s.l. is extremely high compared to ITS differences between the two other well defined *Stuckenia* species: *S. filiformis* and *S. vaginata* (one substitution: McMullan et al., 2011 and our data). Two alternative interpretations are evident. On the one hand, we could assume complete reproductive isolation of populations with different ITS ribotypes. This may be indicated by some populations of *S. pectinata* on the German Baltic Coast, which, even with identical ribotypes, have diversificated strongly enough on microsatellites since their colonization after the last glacial maximum to expect complete absence of gene flow between them (Nies and Reusch, 2005). On the other hand, taking into account extensive interspecific hybridization within *Stuckenia* (Kaplan, 2008), the absence of gene flow between sympatric populations of *S. pectinata* is difficult to imagine. We expect that the observed genetic divergence is caused by local adaptation to contrasting environmental conditions (i.e. salinity, cf. Nies and Reusch, 2005; Triest et al., 2010), combined with the different age of water bodies (Santamaría, 2002), where founder effect also could play a role. Consistent with this expectation plants, tentatively referred to as *S. macrocarpa*, *S. chakassiensis* and *S. pectinata* s.str., grow in sympatry in water bodies with different combinations of age and salinity: fresh young (< 1000 years old) lakes, brackish old (> 10 000 years old) lakes and brackish young (< 7000 years old) water bodies respectively. In this case, introgression between different morphotypes could be substantially diminished by the low competitive ability of hybrids.

There is a striking contrast between the absence of *Stuckenia pectinata* s.l. ITS variability across almost all of Europe (with except for Pechora delta), colonized by only the ribotype **B** and the relatively high ITS variability in southern Siberia. Similar genetic homogeneity across vast ranges of relatively northern (> 40° N) western Europe was revealed for phylogenetically close and ecologically similar *Ruppia cirrhosa* (Petagna) Grande due to recent northwards expansion from the Mediterranean following the Last Glacial Maximum (Triest and Sierens, 2014). This explanation could be also applied to *S. pectinata* s.l. as southern Siberia is characterized by ancient lakes which would accommodate increasing genetic diversity over time (Triest et al., 2010), and had not experienced Quaternary glaciations (Durnikin, 2013; Chepinoga, 2015). Although area of some of the investigated European populations of *S. pectinata* (16, 17, 33–39) were not covered by the glacier during the Last Glacial Maximum, they are also young as they could appear only after the last Pleistocene regression of the Black and the Caspian seas. Finally, existing ancient European populations could be missed, as the sampling in Europe is sparse, even taking into account the published data that are summarized above.

ITS cloning of two Chinese *Stuckenia pectinata* revealed both ribotypes **A** and **B** in one plant (Yang et al., 2016) indicating that the ITS variability within a particular population could be underestimated. However, this finding remains to be verified as ITS cloning in other two Chinese *S. pectinata* samples found no coexisting ribotypes in one plant (Wang et al., 2007). It is also difficult to imagine that only ribotype **B** was revealed in all the European localities (with except for Pechora delta) just by chance. The absence of within-population ITS variability (for which we tested in several localities: Kipriyanova, Bobrov, unpubl.) and the relative rarity of polymorphic sites in ITS sequences, also suggest that coexistence of different ribotypes in one *S. pectinata* s.l. plant is unlikely in Europe. Accordingly, our estimates of relative genetic variability in Europe and Asia are quite robust, although future ITS cloning and (better) application of more reliable low copy nuclear genes (such as PHYB: Yang et al., 2016) would further clarify the taxonomy of the genus.

To conclude, there exists genetic diversification within *Stuckenia pectinata* s.l. in southern Siberian water bodies with different combinations of age and salinity. However, the absence of clear correspondence between genetic variation and the proposed taxonomic differentiation within Siberian *S. pectinata* s.l. does not sufficiently support delimitation of any species (such as *S. chakassiensis* and *S. macrocarpa*) within *S. pectinata* s.l.

Author contributions

LM and AB collected material, LK and SM did the lab work, all the authors analyzed the data and contributed to the manuscript.

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