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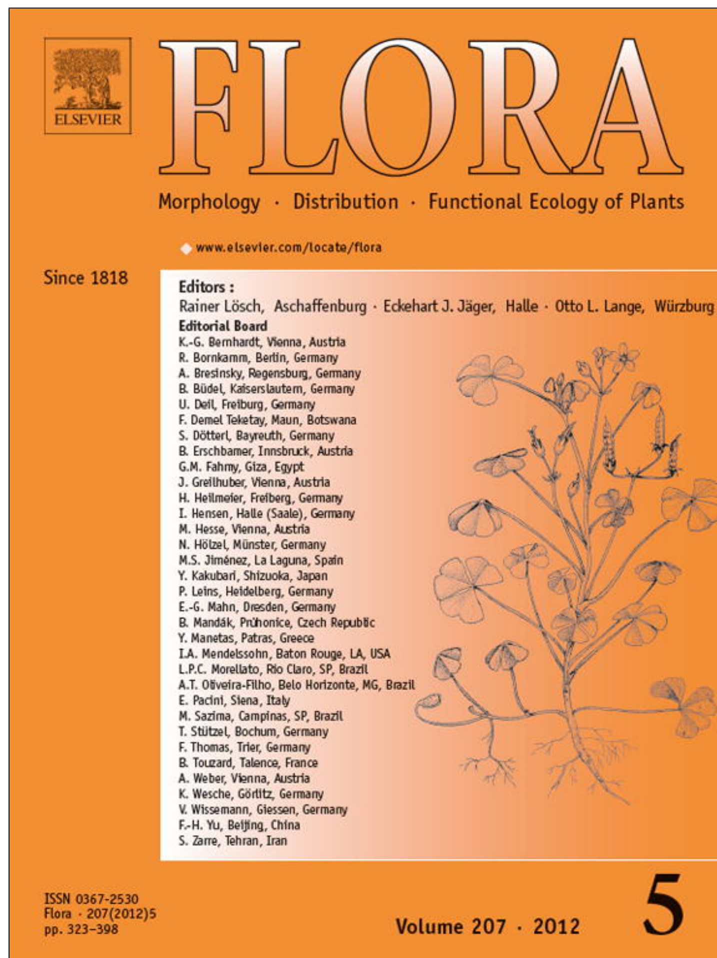

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No sex at all? Extremely low genetic diversity in *Gagea spathacea* (Liliaceae) across Europe

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ABSTRACT

Most vascular plant taxa are able to reproduce vegetatively in addition to sexual reproduction. Some plants even survive despite of (near to exclusive) sexual sterility. However, very few of these taxa are non-apomicts with a wider distribution range.

Here we present the rare case of a virtually sexually sterile, non-apomictic plant, which was able to colonise its Central European range solely by clonal multiplication of a single genotype via subterranean bulbils: the geophyte *Gagea spathacea* (Liliaceae) occurs in forests in northern Germany and adjacent southern Scandinavia, with scattered populations spread all over Central Europe; recently the species was recorded from the Caucasus. We used AFLP fingerprinting to genotype 138 samples from 52 populations throughout this range. The analyses revealed an extremely low genetic diversity (simple matching distances 0–0.1353). By using a threshold for clone identity of <0.02, 136 of 138 samples were assigned to a single clone, the two deviating plants originated from one German population and from the Caucasus. The “megaclone” was present in all analysed Central European populations. A subset of 22 plus four additional populations was studied by DNA sequencing of the ITS region and of *psb*A-*trn*H IGS; these sequences were also found to be highly uniform.

The absent spatial genetic structure throughout the species’ range lends strong evidence that *G. spathacea* is a sexual sterile, nearly monoclonal taxon. Most probably this can be explained by either the high ploidy level (nonaploidy) and/or the assumed hybridogeneous origin of this taxon. However, the purely clonal state with bulbils as sole means of dispersal poses further questions concerning species’ origin and putative colonisation history.

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Introduction

In addition to sexual reproduction, most flowering plant species rely at least to some degree on various modes of vegetative reproduction. Only a small number of plants seems to have completely lost the ability to reproduce sexually (see e.g. Eckert, 2002). Most of these taxa are apomicts that evolved from closely related sexual taxa and now develop seeds asexually.

However, formation of seeds as one of the evolutionary key innovations of flowering plants is rarely abandoned, even if many species additionally reproduce vegetatively by specialised diaspores (like bulbils, turions) or by clonal reproduction through disintegration of genets (Frey and Lösch, 2010; Urbanska, 1992). Plants relying solely on vegetative diaspores seem to be extremely rare (Eckert, 2002; Villarreal et al., 2010). A number of taxa does so in parts of their ranges, like invasive species with only one sex introduced (only male *Elodea canadensis* Michx. introduced in Europe), at the limits of their ranges (Bauert et al., 1998) or within small and/or relict populations (Lynch and Balmer, 2004; Lynch et al., 1998; Peakall et al., 2003; Villarreal et al., 2010). For the few autochthonous sexually sterile plant species which occupy a more than local range, inherent sexual sterility often results from severe problems in meiosis. Examples are taxa with high (especially anorthoploid) ploidy levels (like in triploid *Sedum bulbiferum*: Tsujimura and Ishida, 2008) or of hybridogeneous origin with mixed parental chromosomal sets.

Within a regional survey comparing reproductive modes and resulting spatial genetic patterns of *Gagea spathacea* (Hayne) Salisb. and *G. lutea* Ker.-Gawl. (Liliaceae) in Western Pomerania (Germany), the former taxon showed extremely low genetic variation in the study region (Pfeiffer et al., 2011) and no seed set (Schnittler et al., 2009). *Gagea spathacea* seems to be sterile throughout its range, as reported by Raunkiær (1895–99) and Westergård (1936) for Denmark, Gustaffson (1946, in Raamsdonk,

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1985) for Sweden, and Levichev et al. (2010) for several European countries.

Two explanations were invoked for the nearly complete sexual sterility: the highly anortho-polyploid chromosomal state (∼nonaploidy – Henker, 2005; Levichev et al., 2010; Peruzzi, 2003; Westergård, 1936) and the supposed hybridogeneous origin of the species (Levichev et al., 2010; Mesícek and Hrouda, 1974; Westergård, 1936); both phenomena can severely hamper meiotic division.

The results of the study by Pfeiffer et al. (2011), which failed to detect clonal diversity on a regional scale (∼30 km between populations), triggered the present study, applying AFLP genotyping and DNA sequencing (nrDNA ITS region, cpDNA *psb*A-*trn*H IGS) to analyse samples from populations throughout the distribution range. We aim (i) to assess genetic (clonal) diversity within and between populations and (ii) to detect any spatial genetic structure (SGS), e.g. a differentiation of populations on geographic scales. Such data allow (iii) to test whether sexual sterility is a general trait of the species and (iv) may reveal colonisation history by relating the current distribution pattern to SGS and dispersal features.

Materials and methods

Study species

Gagea spathacea is an ephemeroid spring geophyte nearly confined to natural moist forests; it rarely occurs in semi-natural parks and woodlands. Flowering (from mid April to early May) is scarce (1–3 flowers) and rare, usually only one in a thousand plants flowers. No evidence for apomixis is known for the genus; the species mainly reproduces vegetatively by formation of numerous subterranean bulbils (up to 54 bulbils per plant per year, Schnittler et al., 2009). Thorough searches (including regular control of plants marked at flowering) in several years and many German populations failed to detect fruit and seed set. Even in populations from the Oldenburg region (where seeds were collected for LEDA traitbase data; Kleyer et al., 2008) no seed set was found in 2010 (populations D_Ns08-11; D. Kunzmann, pers. comm.). Another population with documented seed set in the past in the Westerwald/Hesse (Kalheber and Kalheber, 1966) could not be checked; it was most probably destroyed by trenching in the 2000s (Kalheber, pers. comm.). Finally two capsules were discovered in a single population in Mecklenburg-Western Pomerania (map (MTB) 2334/13, R4456800, L5949500 ± 80 m, U. Schlüter, May 2010). One capsule contained one apparently aborted (misshaped) seed, the other a single small seed which failed to germinate. Likewise, in the *G. spathacea* collection in the Botanical Garden in St. Petersburg (Levichev, 2002) three seeds from two capsules detected in 1998 looked insufficiently developed and failed to germinate.

The species is most common in northern Germany, adjacent southern Scandinavia and northern Poland. A few populations are reported from the Netherlands, Belgium, and France to Poland and the Kaliningrad region. Outposts occur in northern Italy, Slovenia, Croatia, Serbia, Romania, and the southern Caucasus (recent find reported by Timukhin et al., 2010). A summary of the known distribution is listed in Levichev et al. (2010) – compare Fig. 1. The species is regarded as "vulnerable" in Central Europe and listed on several national Red Lists (Schnittler and Günther, 1999). With its distribution area centred in Germany, the country has a special responsibility for the species' survival (Ludwig et al., 2007; Welk, 2001), therefore the taxon was listed as one of the species with a particular responsibility of Germany for its worldwide conservation within the biological diversity initiative of the German Federal Agency for Nature Conservation (Bundesamt für Naturschutz, 2011).

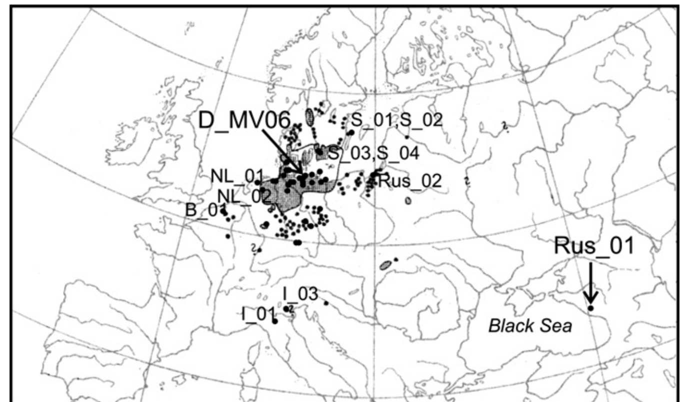

Fig. 1. Geographical locations of the 52 *Gagea spathacea* populations sampled for this study (black dots); the non-German samples are additionally listed with codes as in Table 1; divergent samples are labelled in large print (see Results); background (from Meusel et al., 1965, modified) shows the world range of the species.

Recent infrageneric classifications place *G. spathacea* either in a monotypic sect. *Spathaceae* Levichev (Levichev in Peterson et al., 2008) or incorporate the species in the larger sect. *Didymobulbos* K. Koch (Zarrei et al., 2011). For detailed morpho-anatomical and karyological characters see also Peruzzi et al. (2008).

Sampling

Plant material was collected in spring 2010 from 52 populations covering most of the species' distribution range: Netherlands (NL: 2 populations), Belgium (B: 1), Sweden (S: 4), Italy (I: 2), Russia (Rus: 2) and Germany (D: 41); the latter include three Western Pomeranian populations already analysed by Pfeiffer et al. (2011). Up to 20 plants per population were excavated from different patches within areas of up to ∼100 m^2. In smaller populations, these numbers were reduced, and in extremely vulnerable populations only a few leaves were collected. Populations are coded by abbreviations of the country (for Germany including as well the federal state name abbreviation) and numbered consecutively (Table 1, Fig. 1).

Fresh material (plants with bulbs or leaves) for molecular analyses was stored at −80 °C until DNA extraction, or was dried in paper bags in silica gel; herbarium specimens and samples for sequencing were air-dried. Since we often encountered infections with the smut fungus *Ustilago ornithogali* (Schmidt & Kunze) Magnus (Pfeiffer et al., 2011), we used only bulbs (without roots and outer sheaths) and parts of leaves without visual signs of infection for DNA extraction. For nearly all populations, a herbarium voucher has been deposited in GFW and HAL (Table 1).

***psb*A-*trn*H IGS and ITS region**

In total, analyses of the nrDNA ITS region and cpDNA *psb*A-*trn*H IGS were conducted for 30 samples from 26 populations (Table 1), including 23 samples from 22 populations from the AFLP dataset and seven samples analysed in former studies. DNA was isolated from about 10 mg air dried leaf material with the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. The ITS region (for ITS5 and ITS4 primers see White et al., 1990) and the *psb*A-*trn*H IGS (for primers see Sang et al., 1997) were amplified in a GeneAmp PCR System 9700 (Perkin Elmer). PCR products were sequenced prepared as "u-mixes" (100–200 ng PCR product and 10 pmol sequencing primer) using the StarSEQ® Sequencing Service (StarSeq GmbH, Mainz). Both strands were sequenced at least twice, manually edited and combined into a single consensus sequence. All sequences have been deposited in the EMBL gene bank (for accession numbers see Table 1). Sequence alignment was

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Table 1
Population codes, sampling data, voucher information, no. of genotyped plants (*N*) and EMBL accession numbers for the cpDNA *psbA-trn*H IGS and nrDNA ITS region of the analysed populations of *Gagea spathacea* (sequences generated in former studies are marked by asterisks). Country codes: B – Belgium, D – Germany, I – Italy, NL – Netherlands, Rus – Russia, S – Sweden.

Pop. code	Country, region	Collector(s)	Voucher	*N*	EMBL no. (*psbA-trnH*/ITS region)
B_01	B, Raspaillebos, Geraardsbergen	J. Cornelis	GFW039905, HAL110368	3	FR874122/FR874113
D_Bay01	D, Bavaria, Buchberg	D. Harter	GFW039874, HAL110398	3	–
D_Bay02	D, Bavaria, "Obersau"	M. Schnittler & K. Horn	GFW039875, HAL108202	3	FR691830/FR691811
D_Bay03	D, Bavaria, Tyrolsberg 1	M. Schnittler & K. Horn	GFW039876, HAL110399	3	–
D_Bay04	D, Bavaria, Tyrolsberg 2	M. Schnittler & K. Horn	GFW039877, HAL110400	2	–
D_Bay05	D, Bavaria, Tyrolsberg 3	M. Schnittler & K. Horn	GFW039878, HAL108201	3	FR691831/FR691812
D_Bb01	D, Brandenburg, Gehren	H. Illig	GFW039897, HAL108200	1	FR691832/FR691813
D_Bb02	D, Brandenburg, Höllenberge	H. Illig	HAL110412	1	–
D_Hes01	D, Hesse, Mademühlen	H. Kalheber	GFW039898, HAL108199	3	FR691833/FR691814
D_MV01	D, Mecklenburg-Western Pomerania, Rügen: Putbus	M. Schnittler	GFW039865, HAL110401	3	–
D_MV02	D, Mecklenburg-Western Pomerania, Rügen: Granitz	M. Schnittler	GFW039866, HAL110402	3	–
D_MV03	D, Mecklenburg-Western Pomerania, Groß Stowe	T. Lemke	GFW039867, HAL110403	3	–
D_MV04	D, Mecklenburg-Western Pomerania, Altkalener Wald	P. Bolbrinker	GFW039868, HAL108198	3	FR691834/FR691815
D_MV05	D, Mecklenburg-Western Pomerania, Ludwigslust "Drusenhorst"	U. Schlüter & H. Sluschny	GFW039869, HAL110404	3	–
D_MV06	D, Mecklenburg-Western Pomerania, Züsower Forst	H. Henker	GFW039870;HAL112556, HAL108197	3	A: FR874128/FR691835 B: FR691816/FR874121
D_MV07	D, Mecklenburg-Western Pomerania, Eichholz	E. Schreiber	GFW040641, HAL110405	3	–
D_MV08	D, Mecklenburg-Western Pomerania, Lassahn-Bernstorf	H. Sluschny & U. Schlüter	GFW039871, HAL110406	3	–
D_MV09	D, Mecklenburg-Western Pomerania, Rostocker Heide	S. Leipe	GFW040642, HAL110407	3	–
D_MV10	D, Mecklenburg-Western Pomerania, Starkower Holz	S. Starke (+ AG Geobot.)	GFW039872, HAL110408	3	–
D_MV11	D, Mecklenburg-Western Pomerania, Parchim/Neuklockow	C. Möller	GFW040643, HAL110409	3	–
D_MV12	D, Mecklenburg-Western Pomerania, Markgrafenheide: "Radelsee"	S. Leipe	GFW040644, HAL110410	3	–
D_MV13	D, Mecklenburg-Western Pomerania, Hütter Wohld near Parkentin	S. Leipe	GFW039873, HAL110411	3	–
D_MV_D	D, Mecklenburg-Western Pomerania, Drosedower Forst near Demmin	M. Schnittler, A. Klahr, T. Pfeiffer	–	2	–
D_MV_E	D, Mecklenburg-Western Pomerania, Greifswald: Elisenhain	M. Schnittler, A. Klahr, T. Pfeiffer	–	3	–
D_MV_W	D, Mecklenburg-Western Pomerania, Wrangelsburg	A. Klahr, A. Heinrich	–	1	–
D_Ns01	D, Lower Saxony, Peine: Hainwald	J. Heinken, Th. Heinken	GFW039896, HAL108196	3	FR691836/FR691817
D_Ns02	D, Lower Saxony, Gifhorn: "Der Maassel"	I. Kanth	GFW039895, HAL110413	3	–
D_Ns03	D, Lower Saxony, Hänigsen: "Hellmanns Kohlen"	I. Kanth	GFW039894, HAL110414	3	–
D_Ns04	D, Lower Saxony, Wathlinger Gutsforst	I. Kanth	GFW039893, HAL110415	3	–
D_Ns05	D, Lower Saxony, "Schilfbruch" bei Ketze	I. Kanth	GFW039892, HAL108195	3	FR691837/FR691818
D_Ns06	D, Lower Saxony, Barwedel: Itschenkuhle	J.-H. Schwarz	GFW039891, HAL108194	3	FR691838/FR691819
D_Ns07	D, Lower Saxony, Barwedel: "Wendenwiesen"	J.-H. Schwarz	GFW039889, HAL110416	3	–
D_Ns08	D, Lower Saxony, Oldenburg: "Wold"	D. Schabelreiter	GFW039888, HAL110417	3	–
D_Ns09	D, Lower Saxony, Ammerland, "Mansholter Holz"	D. Schabelreiter	GFW039887, HAL110364	4	–
D_Ns10	D, Lower Saxony, Ammerland, Hankhauser Wald	C. Peppler-Lisbach	GFW039886, HAL110365	3	–
D_Ns11	D, Lower Saxony, Oldenburg, Mansholt	D. Kunzmann	GFW039885, HAL108193	3	FR691839/FR691820
D_SH01	D, Schleswig-Holstein, "Dragonerkoppel Schulenberg" b. Itzehoe	K. Romahn	GFW039881, HAL110366	3	–
D_SH02	D, Schleswig-Holstein, Blochsdorfer Holz b. Westense	K. Romahn	GFW039882, HAL108192	3	FR691840/FR691821
D_SH03	D, Schleswig-Holstein, Westerwerker See b. Glücksburg	K. Romahn	GFW039883, HAL110367	1	–
D_SH04	D, Schleswig-Holstein, Pohlsee b. Langwedel	K. Romahn	GFW039884, HAL108191	3	FR691841/FR691822
D_Th01	D, Thuringia, Weimar: Nohraer Holz	M. Schnittler	GFW039879, HAL108190	3	FR691842/FR691823
D_Th02	D, Thuringia, Suhl: Gehlberg	M. Schnittler	GFW039880, HAL108189	3	FR691843/FR691824
I_01	I, Northern Appenines, Serramazzoni	L. Peruzzi	GFW039906, HAL110362	1	FR874123/FR874114
*I_01	I, Northern Appenines, Serramazzoni	L. Delfini, F. Fiandri, U. Lodesani & C. Santini	CLU17518 Peruzzi et al., 2008	–	AM409347/AM422465
I_03	I, Monte Rua, Colli Euganei	L. Peruzzi	GFW039907, HAL110363	1	FR874124/FR874115
NL_01	NL, Denekamp	J.W. Bielen & J.J. Hofstra	GFW039899, HAL108189	3	FR691844/FR691825
NL_02	NL, Peizermaden	I. Somhorst	GFW039900, HAL110361	2	–
Rus_01	Rus, Northern Caucasus Mts, near Adler	I. Levichev	HAL112557, 73/09 DNA Levichev (LE)	1	FR874129/FR874118
*Rus_01	Rus, Northern Caucasus Mts, near Adler	I.G. Levichev & I.N. Timukhin	63/08 DNA Levichev LE	–	FR691826/FR691808
Rus_02	Rus, SW Kaliningrad	I.Ju. Gubareva	HAL112558	1	FR874130/FR874119
*Rus_02	Rus, SW Kaliningrad	I.G. Levichev	HAL101828 37/04 DNA Levichev (LE)	–	AJ973174/AM422457
S_01	S, Öland, Böda parish	U.-B. Andersson	GFW039902, HAL110357	3	–
S_02	S, Öland, Högby parish	U.-B. Andersson	GFW039901, HAL110358	3	FR874125/FR874116
S_03	S, Schonen, Frueräften forest (Södra parish)	T. Tyler	GFW039903, HAL110359	3	FR874126/FR874117
S_04	S, Schonen, Pålsbo forest (Torrlösa parish)	T. Tyler	GFW039904, HAL110360	3	–
*D_MV_W2	D, Mecklenburg-Western Pomerania, Wrangelsburg	A. & J. Peterson	HAL112555	–	FR874127/FR874120
*D_MV_14	D, Mecklenburg-Western Pomerania, Forst Wittenhagen	S. Starke	GFW036452	–	FR691829/FR691810
*D_MV_15	D, Mecklenburg-Western Pomerania, Demmin	C. Blümel	GFW029819	–	FR691828/FR691809
*D_SA_01	D, Saxony-Anhalt, Altmark	E. Herz	HAL095844 Peterson et al., 2004	–	AJ416369/AJ427541

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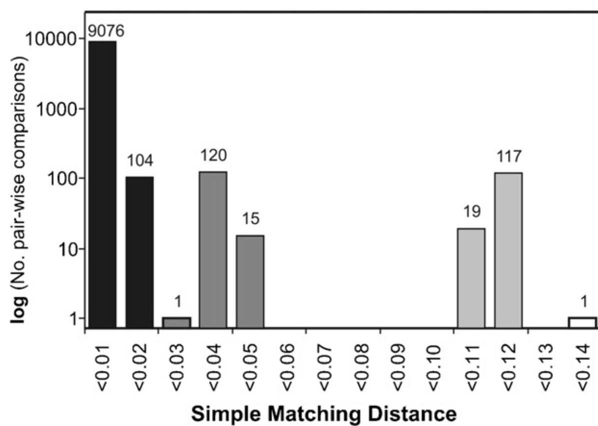

Fig. 2. Histogram of pair-wise comparisons of simple matching distances (only upper class boundaries shown) of 138 *Gagea spathacea* samples analysed with two AFLP primer combinations, drawn to logarithmic scale to highlight the three-modal distribution (figures at bars are numbers of respective pair-wise comparisons). The black bars contain all comparisons within the single recognised megaclone (threshold for identity <0.02, corresponding to 0–4 differences within 269 analysed fragments), bars in medium and light grey represent comparisons of megaclone samples with the individuals D_MV06B or Rus_01, respectively. The white bar depicts the single combination of the latter two samples.

carried out utilising the Clustal-W multiple alignment procedure in Bioedit 7.0.9.0 (Hall, 1999).

AFLP genotyping and analysis of spatial genetic structure (SGS)

DNA extraction and reactions for AFLP fingerprinting followed the protocol described in Pfeiffer et al. (2011) with the exception of partially using silica-dried samples. A total of 138 *G. spathacea* samples with up to four plants per population (indicated by capital letters) were analysed with AFLP fingerprinting using the primer combinations *Eco*RI + AGG (VIC)/*Mse*I + CTA and *Eco*RI + ACA (6-FAM)/*Mse*I + CAG (compare Pfeiffer et al., 2011). A selection of 15 samples was further tested with four additional primer combinations (with different numbers of generated fragments and degrees of polymorphism): *Eco* + ACA (6-FAM)/*Mse* + CTA, *Eco* + AAC (NED)/*Mse* + CGT, *Eco* + ACT (6-FAM)/*Mse* + CTG, and *Eco*RI + AGA (6-FAM)/*Mse*I + GTG (in the following referred to by their selective bases). These selected samples were analysed with the same number of repetitions to calculate error rates for all primer combinations. The subset of samples included the divergent sample D_MV06B (3 repeats) along with two further samples from the same population, D_MV06A (3) and D_MV06C (6), and twelve specimens from populations throughout the sampling area: D_Bay05A (1), D_Bb01A (3), D_MV08B (3), D_Ns03B (1), D_Th01B (8), D_Hes01B (3), D_SH04A (1), NL_01B (4), I_01 (3), S_03A (1), Rus_01 (4), Rus_02 (3).

AFLP fragments were scored with GeneMapper v3.7 (Applied Biosystems) in a semi-automated fashion, using a defined bin set but checking all peaks and evaluating their presence manually: fragment peaks with ≥50 RFU were always scored as "1", lower peaks were either scored as 1 (if clearly defined), 0 (missing) or "x" (doubtful). For all fragments, individual scoring quality was noted from −1 (poor) to 3 (excellent), allowing to use different subsets of fragments in the analyses, i.e. to include, respectively exclude fragments of lower scoring quality. In six samples, data from each 2–4 lab repetitions were compiled and used as consensus AFLP profiles in the analyses (S_01A, B, C, S_02A, D_Bay02C, D_MV01C). This was conducted especially in cases of insufficient DNA quality, for example in some Swedish samples (S_01, S_02) partially rotten after long mail transport.

The obtained AFLP phenotypes were assigned to multi-locus lineages (MLLs, Arnaud-Haond et al., 2007) or clones using an Excel routine. The implemented algorithm allows to vary the maximum number of tolerated fragment differences between phenotypes of the same clone, and assigns all samples to the same clone which are connected to other samples by at least one combination below the defined threshold (Pfeiffer et al., 2011). In most genotyping studies a small tolerance for clonal identity is granted to account for natural divergence (e.g. caused by accumulation of somatic mutations or DNA contamination) as well as for methodological errors (e.g., Arnaud-Haond et al., 2007; Douhovnikoff and Dodd, 2003; Meirmans and Van Tienderen, 2004; Rogstad et al., 2002). We checked this identity threshold against the minimum in frequency histograms of pairwise genetic distances (Douhovnikoff and Dodd, 2003; Meirmans and Van Tienderen, 2004). For the 15 samples analysed with four additional primer combinations we related this threshold also to error rates between lab repeats of identical samples. To assess SGS, the AFLP profiles and genotyping results were contrasted with a spatial distance matrix.

Results

*psb*A-*trn*H *IGS and ITS region*

Samples (30) of the investigated 26 European populations were found with identical *psb*A-*trn*H IGS sequences (267 bp); only one plant (from a population from Saxony-Anhalt not tested with AFLP, specimen HAL095844) showed a 1 bp insertion.

The ITS region had a uniform length of 615 bp in all samples and was also identical except for one mutation site in ITS2 (position 532). At this position instead of "C" a "Y" was detected in three populations from Mecklenburg-Western Pomerania (both investigated samples from D_MV06; D_MV14, D_MV15) and one from the Netherlands (NL_01, for accession numbers see Table 1).

AFLP fingerprinting: genetic diversity

For the two primer combinations AGG/CTA and ACA/CAG analysed for all 138 samples, a total of 183 and 184 fragments of 100–500 nt length were scored, respectively. Different subsets of the scored fragments consistently generated the same three genotypes with only slightly divergent distance measures and thresholds for genotype identity. The following figures are based on a conservative approach, only using peaks of the highest scoring quality 3. In this dataset, 82.6% and 84.7% of the 131 and 138 fragments used for genotyping were monomorphic. From the polymorphic fragments, only 6 (4.4% of all fragments) and 2 (1.5%) differed in more than one sample, respectively. The maximum simple matching distance was 0.1353; but most samples differed only very slightly or not at all (Fig. 2).

For a threshold of as few as one fragment differing between AFLP phenotypes, most samples (136 of 138) were assigned to a single clone (maximum divergence 4 fragments; simple matching distance SM < 0.02, Fig. 2), comprising samples from all analysed populations throughout the distribution range but the Caucasus (Rus_01). Only two samples were recognised as distinct from this "megaclone": Rus_01 and a single German plant (D_MV06B). This latter sample from Züsow near Wismar differed from the two other samples from this population (D_MV06A, C: included in the megaclone) by 10 fragments (SM 0.0373); the smallest distance was observed in comparison with the sample I_03 (SM 0.0299; Fig. 2). The genetically most distant AFLP profile was detected in the sample from the Caucasus (Rus_01), with at least 28 differing fragments compared to the megaclone (SM ≥ 0.1049), and a maximum of 36 (SM 0.1353) compared to D_MV06B (Fig. 2).

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This delimitation of clones is very robust: It reappeared in a similar nested structure [Rus_01 vs. (D_MV06B vs. megaclone)] in analyses including lower peak qualities as well as in the subset of samples analysed for six primer combinations (total 1088 fragments scored, 834 of highest quality analysed), though with different thresholds for recognition of clones in individual primer combinations (not shown). In this analysis, the divergence between repeats of the same sample (i.e. lab and scoring error rate) reached a maximum of 0.0124 simple matching distance (mean 0.0016) for all six primer combinations. Also for individual primer combinations, maximum error rates were usually much smaller than the threshold for identity. Only in two cases one repeat each displayed a slightly larger divergence caused by ambiguous scorings. Even in these cases, the divergence was still smaller than the delimitation between genotypes.

Spatial genetic structure

A spatial genetic structure is virtually absent, since all Central European populations were assigned to a single clone, ranging from S Sweden in the North to N Italy in the South and from Belgium in the West to the Kaliningrad region in the East. This megaclone is identical with the single genet identified in a study in three populations in Mecklenburg-Western Pomerania (99 samples, Pfeiffer et al., 2011).

The sole exceptions were the slightly diverging plant D_MV06B and especially the single specimen from the Caucasus (Rus_01), i.e. from outside the main range of *G. spathacea*.

Discussion

Genetic diversity

According to the sequence data (ITS region, *psb*A-*trn*H IGS) of the analysed 30 samples from 26 populations *G. spathacea* is highly uniform. In contrast, several mutation sites were found in the ITS region of other polyploid *Gagea* species: ten sites in eleven populations of *G. liotardii* (Sternb.) Schult. & Schult.f. (=*G. fragifera* (Vill.) Ehr. Bayer & G. Lopez); five sites in ten populations of *G. lutea* and five sites in ten populations of *G. bohemica* (Zauschn.) Schult. et Schult. f. (data not shown; for chromosome numbers see Peterson et al., 2009).

AFLP fingerprinting, usually offering much more resolution power due to a higher number of analysed markers, also revealed no relevant genetic diversity and nearly no SGS (compare Pfeiffer et al., 2011 for three populations from Western Pomerania). The overall divergence between the analysed *G. spathacea* specimens was remarkably low, irrespective of the primer combination applied. For primers AGG/CTA and ACA/CAG, the threshold of <0.02 simple matching distance for clonal identity resulted in assigning nearly all samples (136 of 138; Fig. 2) to one exceptionally large and widespread clone. This figure is within the range of identity thresholds commonly applied in AFLP studies. In an analysis of the hexaploid *G. lutea* using the same primer combinations, a threshold of <0.05 was applied and revealed a high overall diversity with a mixture of unique genotypes and small clones (Pfeiffer et al., 2011). Although generally such values are not directly comparable between primers or taxa, many analyses used similar thresholds for delimitation of clones (e.g. Arens et al., 2005; Pfeiffer, 2007: 0.02; Vandepitte et al., 2009: 0.03).

The calculated mean error rate of 0.0016 (maximum 0.0124) simple matching divergence between repeats of the same sample calculated from the second dataset also fits well within the range of 0–2% reported by Mueller and Wolfenbarger (1999). It further justifies the applied threshold for clonal identity and indicates that divergence caused by methodological errors and/or somatic mutations is rather small.

A low clonal diversity can either indicate a low genotypic differentiation of the samples; but it might also be due to an insufficient marker resolution failing to detect differences between genets (Arnaud-Haond et al., 2005; Bonin et al., 2004). AFLP fingerprinting relies on analysis of numerous fragments generating multi-locus profiles, which principally enhances resolution power considerably.

For a highly polyploid taxon, minor differences between genets caused by somatic mutations are harder to detect since they first may occur in only a single copy of the polyploid genome. Consequently, new fragments caused by mutations should appear as comparatively small peaks which may blend into the overall background and thus escape scoring. This might be one reason for the great genetic uniformity within the *G. spathacea* megaclone across a large geographic range. However, in the hexaploid *G. lutea* this was not evident (Pfeiffer et al., 2011). We thus assume low clonal diversity to be an inherent feature of *G. spathacea*'s genetic structure.

However, the deviations of the German sample D-MV06B (mean simple matching distance to megaclone including the other two samples from this population 0.0383; single mutation site in ITS2 sequence shared by further samples) as well as of the more divergent Rus_01 (mean distance 0.1121, Fig. 2; no difference in sequence data) are too prominent to be explained by somatic mutations alone, we thus assume them to be caused by extremely rare sexual events.

Dispersal ecology

The dominance of large clones is consistent with the predominantly vegetative reproduction strategy assumed for *G. spathacea* (Levichev et al., 2010; Schnittler et al., 2009). The many subterranean bulbils may be only dispersed within short range by translocation of substrate through tree falls, or through digging or wallowing activities of animals. Levichev et al. (2010) also assume a transport with water streams, which could account for larger dispersal distances.

Generally, most vegetative diaspores have a reduced dispersal potential compared to seeds. However, under suitable conditions individual clones can attain enormous sizes in non-apomicts as well: In *Allium ampeloprasum* L. var. *babingtonii* in SW England Treu et al. (2001) detected only one clone in a RAPD study, just like Tsujimura and Ishida (2008) in Japanese *Sedum bulbiferum* studied by isozymes. Due to the low dispersal potential of the subterranean bulbils, *G. spathacea* is an exception even compared to most of these taxa. We are not aware of another autochthonous clonal flowering plant with such a widely distributed clone, nor do we know of other (nearly exclusively) sterile taxa (morphologically distinct and stabilised species) with such an extensive range in natural habitats (but see Ellstrand and Roose, 1987, suspecting monoclonality in *Gaura triangularis* Buckley).

Is* G. spathacea *sexually sterile?

Even in larger *G. spathacea* populations with (many) flowering plants, seed set is virtually absent (for rare reports see Kalheber and Kalheber, 1966; D. Kunzmann in LEDA traitbase; data on seed and fruit traits in Tomovic and Niketic, 2005). This nearly complete sexual sterility of *G. spathacea* (Levichev et al., 2010; Weeda, 2006; Westergård, 1936) is probably no recent acquisition but inherent in the species' state. Wang et al. (2004) identified different ecological factors accounting for sexual failure in the rare Gesneriaceae *Titanotrichum oldhamii*: (1) poor pollinator service, (2) suboptimal environmental conditions, (3) increased

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inbreeding resulting in low seed set and establishment rates, and (4) resource allocation towards vegetative reproduction (bulbils and rhizome development) rather than fruits. Since *G. spathacea* is the latest flowering of the German *Gagea* taxa, the first two factors are probably of minor importance. However, especially resource allocation patterns may affect *G. spathacea*: the high number of bulbils produced per year limits investments into the parent bulb, hence only a very small proportion of the plants can accumulate sufficient resources for flowering (Schnittler et al., 2009).

However, the two most plausible (non-exclusive) explanations for the sterility of *G. spathacea* are grounded in the species biology: the odd and extremely high ploidy level and the assumed hybridogeneous origin (Levichev et al., 2010; Mesícek and Hrouda, 1974; Westergård, 1936). Both conditions can pose an inherent obstacle to meiotic division and are known to restrict sexual reproductive success (cf. Westergård, 1936; compare Peterson et al., 2010 for clonal 5× *G. bohemica* in Bohemia and Wales).

Hybridisation is generally common within *Gagea* (e.g., Peruzzi, 2008; Peruzzi et al., 2008; Peterson et al., 2008, 2009, 2011). For *G. spathacea*, Levichev et al. (2010) suggest an ancient hybrid origin resulting from an intersectional or even intergeneric cross, but give no hint towards putative parental taxa. Incongruent trees based on ITS and cpDNA data (Peruzzi et al., 2008, 2011; Peterson et al., 2004, 2009, 2011) could indicate such a hybridogeneous origin, but also provide no information about putative parents for the investigated species.

If *G. spathacea* is a hybrid, the species should be derived from few or even a single interspecific cross (as already hypothesised by Westergård, 1936, pp. 447–448). A multiple hybrid origin would imply a considerable initial genetic variability which should be conserved by further vegetative multiplication. Assuming sexual sterility, this initial diversity may change through selection of individual genets (decreasing diversity, Honnay and Bossuyt, 2005) or somatic mutations (slightly increasing diversity, see Ellstrand and Roose, 1987). Both mechanisms can be of relevance for colonisation history and population structure (compare Kimpton et al., 2002). However, the dominance of one *G. spathacea* clone present throughout the main range in Central Europe and the absent SGS are explained best by a singular hybrid origin. This is feasible since due to the mixed chromosomal states often only very few primary hybrids are viable and able to start a population or, in the long run, to become the ancestor of a stabilised new taxon like *G. spathacea*.

The high and anorthoploid state of *G. spathacea* (102–108 chromosomes, i.e. roughly complying with nonaploidy) is also restricting fertility: Westergård (1936) documented irregular formation of gametes in *G. spathacea* and doubted the capability of normal sexual reproduction in Danish populations. He did not exclude parthenogenesis, but since (i) this phenomenon is unknown in the genus and (ii) seeds are extremely rare, this seems to be unlikely. However, even a few asexual seeds would significantly increase the colonisation spread of a clone since they should disperse more readily (through boleochory, myrmecochory) than subterranean bulbils.

Despite of the karyological facts severely impeding sexual reproduction, the species may not be completely sterile. Along with irregular pollen, Westergård (1936) also detected few superficially "normal" grains, which may under exceptional circumstances meet viable egg cells. The divergent sample D_MV06B within the main distribution range (co-occurring with the megaclone) may represent such a sexually derived new genet. However, we must assume that such extremely rare events fail(ed) to restore sexuality and are thus of no general evolutionary significance.

Distribution and possible colonisation history

The range of the detected megaclone (virtually identical with the species' range) covers mainly woodlands in regions glaciated during the Pleistocene, hence populations must have established afterwards (Weeda, 2006). Two scenarios are conceivable: (1) The species is of postglacial origin and developed (most probably after a singular hybridisation event) somewhere in the present main range and started to spread into adjacent regions. (2) The species' origin predates the Holocene; it must hence have survived in a glacial refuge, e.g. in a nunatak in the region, or originated there by hybrid speciation. So far, we do not know putative parents, which may be extinct or migrated to regions distant from extant *G. spathacea* range. After the glaciations, one or very few clones started to migrate into the bare regions, with one genet being the most successful coloniser and hence becoming the dominating megaclone. For Pomerania (NW Poland), Popiela (2004) assumes an invasion starting in the late Boreal for *G. spathacea* as well as for the co-occurring (but fully fertile) *G. lutea*.

Together with the distribution centre in northern Central Europe, the high genetic uniformity renders unlikely the speculation of Levichev et al. (2010) that the species originated in the late Miocene in Asia Minor. Within such a time-frame and geographic scale, at least some somatic mutations should have accumulated, leading to an (at least weak) SGS. In addition, the Caucasus population is clearly an outpost of the current range, and it would be hard to explain why the species later colonised nearly exclusively glaciated areas. Irrespective of the time frame, the purely vegetative spread covering total distances of at least 1500 km (between Italy and Sweden) by means of subterranean bulbils is extraordinary.

Conclusion

The dominance of a single megaclone extending through the Central European distribution range together with virtually absent SGS in fingerprinting and sequencing data are best explained by a singular hybrid origin and subsequent vegetative spread of one successful hybrid evolving into the distinct, stabilised species *G. spathacea*. As such, this taxon can be regarded as a rare "evolutionary accident" with surprisingly efficient vegetative multiplication by bulbils granting survival and (at least local) spread.

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