Reproductive isolation vs. interbreeding between *Gagea lutea* (L.) Ker Gawl. and *G. pratensis* (Pers.) Dumort. (Liliaceae) and their putative hybrids in Mecklenburg-Western Pomerania (Germany)

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Abstract

Mecklenburg-Western Pomerania is the main range of two hybridogeneous *Gagea* taxa, *G. megapolitana* Henker and *G. pomeranica* Ruthe, which have the same two parental species, *G. lutea* (L.) Ker. Gawl. and *G. pratensis* (Pers.) Dumort. We assessed the degree of reproductive isolation vs. interbreeding between these taxa using data from field observations, crossing experiments and pollen characteristics for nine hybrid, four *G. lutea* and five *G. pratensis* populations. Pollen viability was highest in 6x *G. lutea* and lowest in *G. pratensis* (most probably 5x), with intermediate figures for the studied hybrids (5x–7x). Despite the assumed anorthoploid states, sexual reproduction (though sometimes very rare) was recorded for all populations in the field and/or in experiments. The crossing experiments revealed that all taxa are also able to hybridize. However, there were differences in the directions (i.e. role as maternal vs. paternal parent) as well as the success of the crossings: the primary hybridization *G. lutea x G. pratensis* resulted in more seeds than the reverse combination (17.5 vs. 3.3%), but the seed set was highest in backcrosses of the hybrids with *G. lutea* pollen (41.2%). These differences can be explained by overlap of flowering times, and reduced fertilities due to ploidy levels. The study showed that the taxa of the *G. lutea-pratensis* hybrid complex are not yet reproductively isolated but can interbreed and will generate hybrids of higher ranks, forming a hybrid swarm, most probably leading to introgression via backcrosses with *G. lutea*.

Keywords: aneuploidy, crossing experiment, hybridization, polyploidy, sexual sterility.

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Introduction

There are two recognized key processes for plant speciation: hybridization (reviews by Hegarty & Hiscock 2005; Chapman & Burke 2007; Soltis & Soltis 2009) and polyploidization (e.g. Hegarty & Hiscock 2008; Soltis *et al.* 2009). Prominent examples are given in Slotte *et al.* (2008) and Hersch-Green and Cronn (2009) (compare review by Rieseberg & Willis 2007).

For the species-rich genus *Gagea* Salisb. of the Liliaceae, polyploidy was confirmed and a hybrid status was already assumed by early investigators for *G. pomeranica* Ruthe (Pascher 1904), *G. spathacea* (Hayne) Salisb. (Westergård 1936) and *G. liotardii* (Sternb.) Schult. & Schult.f. (Bianchi 1946, as *G. fistulosa* Ker-Gawl.). Recent studies have highlighted the relevance of both processes for speciation in the genus (Peruzzi 2008b; Peterson *et al.* 2009, 2011; Zarrei *et al.* 2012): combined morphological, karyological and molecular analyses provided evidence for the hybridogeneous origin of, e.g. *G. microfistulosa* Levichev (*G. villosa* Sweet *x G. fragifera* [Vill.] E.Bayer & G.López), *G. polidorii* J.-M.Tison (cf. opposite cross; Peterson *et al.* 2009), as well as *G. megapolitana* Henker and *G. pomeranica* (both *G. pratensis* [Pers.] Dumort. *x G. lutea* [L.] Ker Gawl.; see below).

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The latter two *Gagea* species of hybrid origin have their main distribution in Mecklenburg-Western Pomerania and were named accordingly: *G. pomeranica* (Ruthe 1893) and *G. megapolitana* (Henker 2005), respectively. For the former taxon the hybridogeneous state (listing *G. lutea* and *G. pratensis* as putative parent species) was already assumed by some early investigators (Ruthe cit. in Pascher 1904 p. 114, although still doubted in the original description by Ruthe 1893). Molecular studies have confirmed this hybrid origin (Peterson *et al.* 2004a, 2008, 2009; Peruzzi 2008b); and the same two species were also identified as parents of *G. megapolitana* (Peterson *et al.* 2009). In all specimens of both taxa sequenced so far, only *G. pratensis* cpDNA-haplotypes were present, and with maternal inheritance of plastids (Bohdanowicz & Lewandowska 1999 for *G. lutea*), this species is most likely the 'mother' of both hybrid taxa. However, probably different (rare 6*x* vs. 4*x*) cytotypes of *G. pratensis* were involved in the initial formation of *G. megapolitana* and *G. pomeranica*, respectively (Peterson *et al.* 2009). The occurrence of different cytotypes within a species is quite common for the genus in general (e.g. Peruzzi 2008a) and for this species complex: according to chromosome counts (see Henker 2005 and references therein), *G. lutea* is strictly hexaploid with 2n = 72 chromosomes. Populations of the other taxa showed varying ploidy levels, including anorthoploid (and partly also aneuploid) states. The figures are interpreted as typically 6*x* (2n = 72), rarely 7*x* (2n = 84) in *G. megapolitana*, most often 5*x* (2n = 60), rarely 6*x* (2n = 72) in *G. pomeranica*, and 4–6*x* in *G. pratensis* (all seven populations counted from Mecklenburg-Western Pomerania had pentaploid chromosome sets), respectively (Henker 2005).

Generally, anorthoploidy and hybridogeneous, mixed chromosomal sets alike can pose an obstacle for meiotic division and hence limit or even prevent sexual reproduction (e.g. Hegarty & Hiscock 2005, 2008). However, despite sexual failure, plant taxa can persist (e.g. *Wollemia nobilis*, Peakall *et al.* 2003) and even spread if they have additional means of vegetative or agamospermous reproduction (e.g. Ellstrand *et al.* 1996). This also accounts for the genus *Gagea* (Peruzzi 2008b), where nearly all species are able to reproduce by bulbils (Levichev 1999). Several species are reported to be virtually sterile, including triploid *G. granatellii* Parl. (Gargano *et al.* 2007), at least some cytotypes of *G. bohemica* (Zauschn.) Schult. & Schult. f. (2*x*–6*x*; compare Jakab & Molnár 2011; but see Peterson *et al.* 2010), and nonaploid *G. spathacea* (Pfeiffer *et al.* 2012).

Likewise, the fertility of *G. pratensis* seems to be extremely reduced, at least in Mecklenburg-Western Pomerania (Henker 2005), whereas *G. lutea* is fully fertile and sets seed readily (Henker 2005; Schnittler *et al.* 2009). In this study, we analyze reproductive isolation vs. inter-change between the taxa using a combination of field observations and pollination experiments. Specifically we ask:

- Are *G. lutea, G. pratensis* and the hybrids from natural populations in Mecklenburg-Western Pomerania (at least partially) fertile, i.e. do they (i) produce viable pollen and (ii) set fruits and seeds? Furthermore, are the species autogamous?
- Can *G. lutea* and *G. pratensis* be crossed to produce primary hybrids experimentally? And is there really a preference of *G. pratensis* as pollen acceptor, i.e. 'mother,' in crosses?
- Are the hybrids reproductively isolated from each other and/or from their parent species, i.e. do crosses and backcrosses yield normal seeds?

Materials and methods

Study species

Gagea lutea and *G. pratensis* (both placed in section *Gagea*) are the parent species of *G. megapolitana* and *G. pomeranica* (Peterson *et al.* 2009). The morphological determination of the hybrid taxa (Henker 2005) proved to be problematic, hence we refrained from this delimitation and sampled these taxa without prior assumption as 'hybrids.'

Gagea lutea occurs throughout Europe and Eastern Asia, *G. pratensis* extends through Central Europe to Asia Minor, and is more common in the eastern (moderately continental to suboceanic) than in the western parts of its range (the species may have colonized this part mainly due to anthropogenic influence creating open landscapes). According to present knowledge the hybrids have much more restricted ranges with an apparent distribution center around Mecklenburg-Western Pomerania. *Gagea pomeranica* is further known from a few localities in (south-)eastern Germany, southern Sweden and the Czech Republic; records from Italy remain doubtful (Henker 2005). *Gagea megapolitana* has only recently been described; according to the known distribution range the taxon is restricted to two regions in northwest Mecklenburg and northeast Western Pomerania (Henker 2005). However, findings from other regions may be added when the taxon is recognized in local floras. Apart from *G. lutea* inhabiting deciduous forests, most populations of the other taxa are found in habitats with strong anthropogenic influence, like cemeteries, church or manor yards, roadside banks, meadows or arable fields.

In the studied *Gagea* spp. flowers are proterogynous (pers. obs., Graebner & Kirchner 1934). In the study region, anthesis occurs from March to April, with *G. lutea* the first and *G. pratensis* the last to flower, and hybrid populations assuming an intermediate state.

Sampling

Plants with young flower buds were carefully excavated in early spring 2008, transferred to the Botanical Garden Greifswald and potted. In total, 765 plants were collected, mainly from populations in Mecklenburg-Western Pomerania: 410 hybrids from nine locations, 200 *G. lutea* plants from four populations, and 155 *G. pratensis* from five locations, including three populations from Central Germany. Most populations of the hybrids and *G. pratensis* are from semi-natural habitats like church or manor yards, meadows and field margins, whereas *G. lutea* was typically collected from deciduous woodlands or parks. For each hybrid population, vouchers of three plants were deposited in the herbarium Greifswald (GFW).

Seed set in the field

Wherever possible, seed set was determined in the field. However, since the main sampling was carried out before the flowering phase and many habitats are later mown, data (Table 1) are based on (i) plants studied during later visits in 2008 from six hybrid populations and (ii) capsules collected in 2009 and 2010 in four populations in the course of other studies (cf. Harter 2008; Formella 2009). The capsules were dried in paper bags, then the seeds were checked under a binocular and classified as 'normal' or 'aborted' (i.e. abnormal size and/or irregular form, or obvious damage). In 2008, the mere presence of normal seeds was recorded; for the fruits collected in hyb1 and the two populations sampled in 2009, seeds were also counted. Data on fruit set in *G. lutea* and *G. pratensis* were determined in similar fashion.

Crossing experiments

Crossing experiments were performed in spring 2008 to assess the success of (i) autogamous pollination vs. (ii) allogamous intraspecific fertilizations (except for *G. lutea*). We further tested for (iii) interspecific hybridization between the parents *G. pratensis* and *G. lutea* (i.e. creation of primary hybrids, with both taxa as either pollen donor or acceptor), as well as (iv) backcrosses of the hybrids with *G. lutea*. Backcrosses with *G. pratensis* could not be carried out due to insufficient flowering material of the latter species. If possible, all pairings were repeated at least five times for four different pollen acceptor populations ($n \geq 20$, see Fig. 2 for number of crosses). In 2009, some experiments were repeated with *G. lutea* pollen from various sources, pollinating 20 *G. pratensis* plants in the Botanical Garden and backcrossing 142 potted hybrids from all nine populations in the greenhouse.

For the experiments in 2008, the flowering times of the taxa were synchronized by growing them in cold or warm conditions in a climate chamber, respectively. In 2009, the cold spring caused a natural overlap of flowering times. In all pollen acceptor plants, anthers were excised before opening. Pollination was conducted manually in all crosses, using pollen from freshly opened anthers. The pollinated plants were kept in perforated plastic bags (permeable to light and air) in an aerated greenhouse until seed set to prevent contamination with foreign pollen. Primary hybridizations in 2009 (*G. pratensis* x *lutea* pollen) were carried out under field conditions in the Botanical Garden, but bagging the plants after pollination. Mature, still closed capsules were harvested and stored in paper bags to collect seeds after opening. The seeds were classified as 'normal' or 'aborted' as described above.

Germination

Germinability was tested for seeds from the crosses in 2008 (pooled per experiment) and also for the normal seeds collected in the field in hyb1. All normal seeds were sown in pots in the Botanical Garden; seedling emergence was controlled in May 2009.

Pollen data

The viability of pollen grains was assessed indirectly using a fluorochromatic technique (FCR, Kearns & Inouye 1993). Pollen grains were removed from anthers just after opening from two to three plants from each of the 18 populations and incubated for 10 min in a 10% saccharose solution containing a few drops of fluorescein-diacetate in acetone (2 μg/mL). Using an Olympus IX50 reverse microscope with a U-RFL-T fluorescence lamp (excitation 480 nm), at least 300 pollen grains per population were checked for the yellow-green color of fluorescein (used as an equivalent for viable grains) and photographed with fixed scales. For living pollen grains, length and width were determined with analySIS 3.1 LabFlow software (Soft Imaging Systems, Münster, Germany; at least 10 measurements per population). Both pollen viability and size data were pooled for populations and taxa. Data for taxa were analyzed for statistical significance with R (R Development Core Team 2011) using Kruskal-Wallis tests (nonparametric ANOVA) and Mann-Whitney U *post hoc* tests for individual comparisons between taxa, respectively.

Results

Seed set in the field

From 141 plants from six hybrid populations studied in 2008, 32 developed fruits, 21 of them with normal (fertile) seeds. However, the latter originated from only two popu-

Table 1 Compiled data on seed set in the field, pollen size and viability, seed set in experiments (pooled for different crossings of each population) and ploidy level (Henker 2005) for nine *Gagea* hybrid populations (hyb1—Altenkirchen/Rügen, hyb2—Dargun, hyb3—Gingst/Rügen, hyb4—Griebenow, hyb5—Semlow, hyb6—Stolpe, hyb7—Trent/Rügen, hyb8—Vitense, hyb9—Zirkow/Rügen), four populations of *G. lutea* (lut1—Behrenhoff, lut2—Greifswald, Elisenhain, lut3—Greifswald, new cemetry, lut4—Griebenow) and five *G. pratensis* populations (pra1—Behrenhoff, pra2—Wrangelsburg; pra3—Harsleben, Saxony-Anhalt, pra4—Hornhausen, Saxony-Anhalt, pra5—Mühlburg, Thuringia)

Taxon	*Gagea* hybrid								
Code	hyb1	hyb2	hyb3	hyb4	hyb5	hyb6	hyb7	hyb8	hyb9
Field: % plants with fertile seeds 2008 (n)	25.0 (24)	n.d.	62.5 (24)	n.d.	0 (52)	n.d.	0 (14)	0 (14)	0 (13)
Field: mean seeds/capsule (n capsules)	0.69 (42)	n.d.	n.d.	8.38 (34)	0.02 (426)	n.d.	n.d.	n.d.	0.29 (7)
Measured pollen grains	15	22	33	32	55	46	31	45	21
Pollen length ± SD (μm)	82.3 ± 9.6	81.2 ± 9.9	79.6 ± 11.7	80.0 ± 9.5	71.2 ± 8.3	70.7 ± 8.8	80.4 ± 8.5	66.3 ± 9.1	68.9 ± 6.0
Pollen width ± SD (μm)	55.3 ± 3.6	56.4 ± 3.1	55.9 ± 5.4	58.5 ± 5.0	51.5 ± 4.2	54.1 ± 4.4	59.3 ± 3.9	51.7 ± 3.7	52.3 ± 3.9
Pollen viability % ± SD (n pollen)	19.9 ± 3.4 (545)	52.5 ± 27.1 (390)	51.4 ± 21.5 (362)	43.9 ± 10.3 (482)	40.0 ± 6.9 (510)	54.9 ± 4.1 (380)	37.8 ± 12.2 (569)	48.7 ± 9.9 (404)	17.3 ± 9.8 (421)
Seed set in experiments: mother/father	No/no	Yes/yes	Yes/yes	Yes/yes	Yes/yes	Yes/yes	Yes/no	Yes/no	Yes/yes
Sterile fruits in experiments	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	(No)
% Seedling plants in backcrosses 2009 (n plants)	0.0 (7)	58.8 (17)‡	13.6 (22)‡	40.0 (20)‡	28.6 (14)	62.5 (16)‡	83.3 (18)	29.6 (27)	100 (1)
Ploidy level (Henker 2005)	7x	n.d.	n.d.	6x	5x	n.d.	n.d.	n.d.	n.d.

Taxon	*Gagea lutea*				*Gagea pratensis*				
Code	lut1	lut2	lut3	lut4	pra1	pra2	pra3	pra4	pra5
Field: % plants with fertile seeds 2008 (n)	100 (49)	100 (104)	n.d.	n.d.	0 (23)	1.1 (93)	n.d.	n.d.	n.d.
Field: mean seeds/capsule (n capsules)	9.23 (73)	11.64 (259)	10.58 (50)	n.d.	0 (49)	0.10 (164)	n.d.	n.d.	n.d.
Measured pollen grains	32	30	44	14	18	12	24	24	28
Pollen length ± SD (μm)	83.2 ± 6.2	76.1 ± 7.5	82.2 ± 8.4	76.4 ± 6.3	56.6 ± 3.2	65.9 ± 5.7	71.9 ± 6.1	73.9 ± 8.6	58.2 ± 8.5
Pollen width ± SD (μm)	58.5 ± 2.7	56.5 ± 3.2	57.6 ± 3.3	56.9 ± 3.4	43.8 ± 3.8	49.8 ± 2.2	51.1 ± 5.3	56.5 ± 4.9	47.7 ± 5.8
Pollen viability % ± SD (n pollen)	62.6 ± 13.3 (428)	81.1 ± 7.7 (315)	61.3 ± 5.5 (722)	37.6 ± 27.5 (364)	15.8 ± 10.0 (428)	7.8 ± 3.1 (407)	39.9 ± 9.7 (298)	35.5 ± 3.8 (306)	14.2 ± 10.2 (622)
Seed set in experiments: mother/father	Yes/yes	Yes/yes	Yes/yes	Yes/yes	Yes/yes	No/yes	Yes/yes	No/yes	No/yes
Sterile fruits in experiments	Yes†	No	No	No	Yes	n.d.	(No)	n.d.	n.d.
% Seedling plants in backcrosses 2009 (n plants)	—	—	—	—	—	—	—	—	—
Ploidy level (Henker 2005)	n.d., but species generally 6x				Most likely 5x				

†Only in one plant from a cross with *G. pratensis* pollen. ‡In backcrosses 2008 with 10 crossed plants per population and specific *G. lutea* pollen: 40% (hyb2), 90% (hyb3), 0% (hyb4), 30% (hyb6). n.d., not determined.
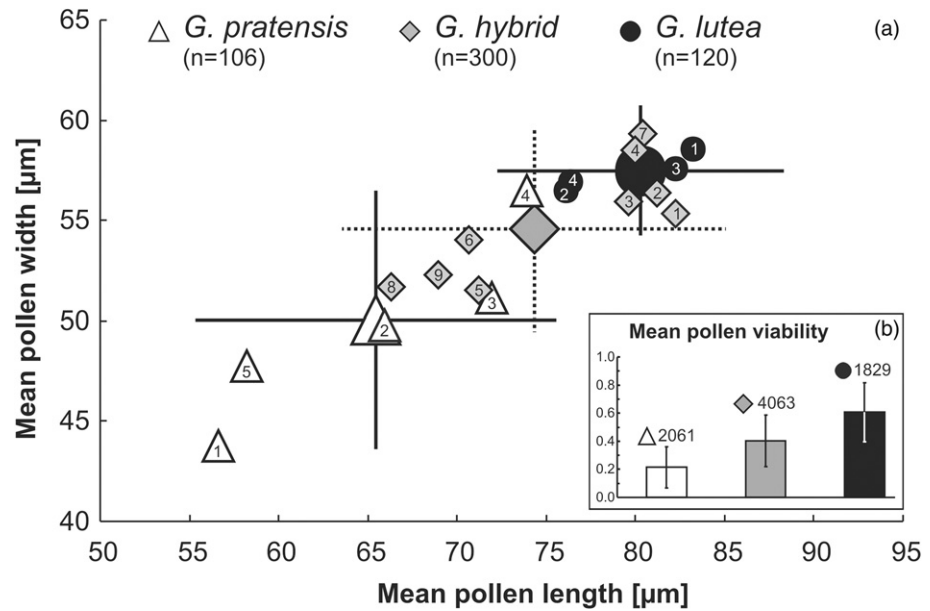
Fig. 1 (a) Mean pollen length and width (± SD) for *G. pratensis* (white triangles), *G. lutea* (black circles) and hybrids (gray diamonds; large symbols). Small symbols depict the means for the investigated populations (see Table 1 for population codes). (b) Proportion of viable pollen grains (± SD) for *G. pratensis, G. lutea*, and hybrid populations. n = number of examined pollen grains per taxon.

lations, hyb1 and hyb3; no fruit set was recorded for hyb5 and hyb9, and the fruiting plants from populations hyb7 and hyb8 contained no normal seeds (Table 1).

The 509 fruits collected in four hybrid populations yielded 323 normally developed seeds, but with major differences in seed set between populations: On average 8.4 normal seeds/capsule were formed in hyb4 compared to < 1 in hyb1 and hyb9 and nearly absent seed set in hyb5 (Table 1), respectively. However, the 29 seeds from hyb1 gave rise to 11 seedlings (germination rate 37.9%; the other seeds collected in 2009/2010 were not tested for germinability).

In *G. lutea*, seed set occurred regularly in all localities investigated (Table 1, Schnittler *et al.* 2009). In contrast, the *G. pratensis* populations from Mecklenburg-Western Pomerania nearly failed to set seed (Table 1), in pra2 a single fruiting specimen was found (no data on seed numbers, Harter 2008).

Pollen viability and size

The size of viable pollen grains differed between but also within the taxa studied. On average *G. lutea* had the largest pollen grains and *G. pratensis* the smallest, whereas hybrids displayed intermediate figures for length and width (Fig. 1a). These differences are highly significant (Kruskal-Wallis values for pollen length [$\chi^2 = 96.0894$] and width [$\chi^2 = 94.7544$]: each d.f. = 2 and $P < 2.2 \times 10^{-16}$, also $P < 10^{-7}$ in all Mann-Whitney U *post hoc* tests between taxa, not shown). However, especially in the latter two taxa large differences were obvious between populations. While the *G. pratensis* records covered a ± continuous range, the hybrid data fell into two groups

with mean pollen sizes of some populations (hyb1–4, 7) being rather similar to *G. lutea* average vs. pollen from hyb5–6, and hyb8–9 being smaller and more similar to *G. pratensis* measures, respectively (Fig. 1a).

Pollen viability showed trends similar to pollen size (Fig. 1b, Table 1): *G. pratensis* pollen was least viable (mean 21.4%, for the two Western Pomeranian populations pra1, 2 only 15.8% and 7.8%); pollen of *G. lutea* performed best (> 60% viable grains for all but lut4). The hybrid populations tested showed intermediate viabilities (always < 55%, mean 40.7%, Fig. 1b, Table 1). A Kruskal-Wallis test revealed the differences to be significant (KW $\chi^2 = 15.3502$, d.f. = 2, $P < 0.0005$), just like all comparisons between the three taxa in Mann-Whitney U *post hoc* tests ($P \leq 0.0149$).

Crossing experiments

The conducted artificial pollinations yielded at least some seed set in nearly all combinations tested (Table 1). Capsules in both *G. pratensis* and the hybrids often contained aborted or irregularly shaped seeds (not included in the data in Fig. 2), some fruits had even no seeds at all (observed only once in *G. lutea*, Table 1). Generally, all taxa are self-compatible, but allogamous seed set is usually higher (Fig. 2). Hybridizations between *G. lutea* and *G. pratensis* resulted in primary hybrids formed by both *G. lutea* and *G. pratensis* mother plants, respectively, with better seed set observed on the former species (Fig. 2). All crosses of *G. lutea* mother plants with *G. pratensis* pollen from four populations yielded at least some seed set and seedlings (Table 2); the most successful cross was with pollen from pra4 in Central Germany, where four of five plants developed fruits with a total of 44 normal seeds
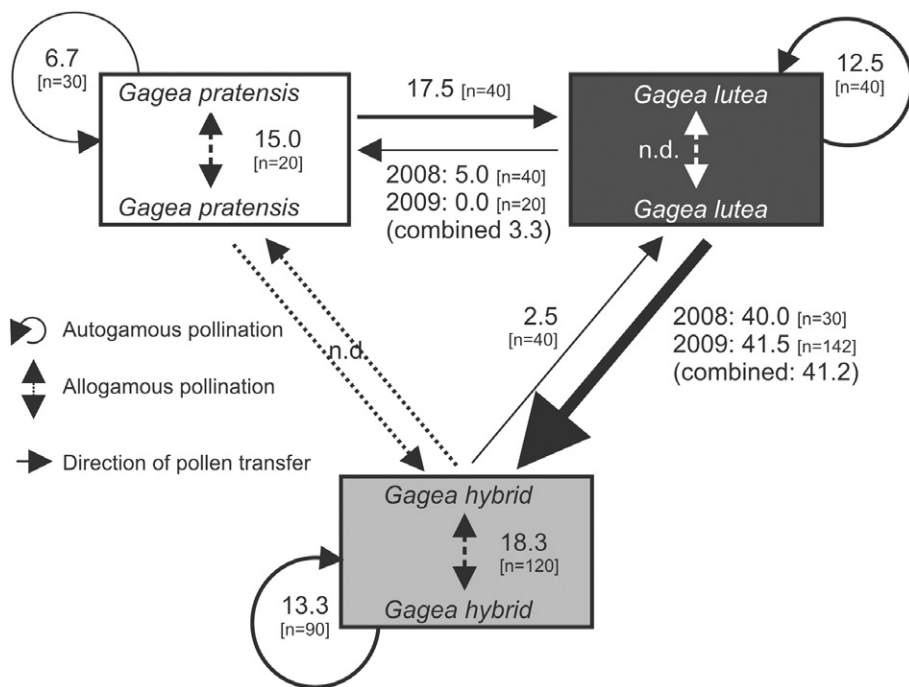
Fig. 2 Results of the crossing and hybridization experiments in the *Gagea lutea-pratensis* hybrid complex (data pooled for taxa). Arrow heads indicate the direction of pollen transfer; with solid straight arrows for interspecific and dashed ones for allogamous pollinations; curved arrows represent results for autogamous selfing. The numbers at arrows are percentages of plants with normally developed seeds in the respective experiments. n.d., allogamous crosses in *G. lutea* not determined, but fully fertile (compare Schnittler *et al*. 2009); backcrosses of hybrids with *G. pratensis* not conducted.

Table 2 Percentage of deformed seeds and germination rates of normal seeds from crossing experiments (in parentheses: numbers of seeds tested for germination)

Pollen acceptor taxon ('mother')	*Gagea* hybrid		*Gagea lutea*		*Gagea pratensis*	
Experiment	Aberrant seeds (%)	Germination rate	Aberrant seeds (%)	Germination rate	Aberrant seeds (%)	Germination rate
Autogamy	25.8	34.5 (110)	19.3	30.5 (131)	0.0†	80.0 (10)†
Allogamy	6.7	57.9 (159)	n.d.	n.d.	40.0	16.7 (6)‡
Primary hybridization	—	—	14.5	29.2 (65)	81.0	25.0 (4)‡
Backcross with *G. lutea*	28.8	63.0 (270)	0.0	72.2 (18)§	—	—

†Seeds only from population pra3 (not tested in other experiments due to lack of material). ‡Only from population pra1. §Only from population hyb2. n.d., experiment not conducted.

(ten of which germinated successfully). The opposite cross *G. pratensis x lutea* was much less successful, yielding three fruiting plants from pra1 with only four normal seeds but one seedling (Table 2), and no seed at all in mothers from three other populations in 2008 and in the Botanical Garden in 2009 (Fig. 2).

Likewise, backcrosses between the hybrids and their putative parent *G. lutea* were successful: fruit and seed set occurred in all combinations tested where hybrid plants were pollinated with *G. lutea*, with similar ratios in both years (Fig. 2; higher than in the hybrid allogamous crosses). All but the hybrids from population hyb1 developed at least a few to many (275 from 17 crossed plants from hyb2) normal seeds (see Table 1 for seed set rates of individual populations in 2009 with mixed *G. lutea*

pollen). The opposite backcross of *G. lutea x* hybrid was only successful in one plant from population hyb2 (Fig. 2), but the single capsule produced 18 normal (and no deformed) seeds. Despite the difference in sown seed numbers, germination (only tested for 2008 experiments) was rather high for both types of backcrosses (63.0 and 72.2%, Table 2).

Generally, formation of normal seeds in crosses was lowest in *G. pratensis* (Fig. 2); and germination was also poor apart from autogamous seeds from population pra3 from Central Germany (only tested in this cross; Table 2). In the hybrids as well as in *G. lutea*, the percentages of deformed seeds were lower, and germination rates were higher in allogamous and interspecific than in autogamous crosses (Table 2).

Discussion

Reproductive success and ploidy

The morphological treatment of Henker (2005) showed that the taxa of the *Gagea lutea-pratensis* hybrid complex are rather variable with regard to ploidy level ($4x$–$7x$, in Mecklenburg-Western Pomerania $5x$–$7x$) and bulb and bulbil morphology (compare specimen scans in Henker 2005). While *G. lutea* and *G. pratensis* are readily distinguished morphologically and by their reproductive strategies, we often encountered problems in assigning plants to the described hybrid taxa. Even some of the individuals from hybrid populations cited as *G. megapolitana* (hyb1, 4) or *G. pomeranica* (hyb5; Henker 2005) had intermediate character states impeding a reliable identification. In species complexes including hybrids (and especially reticulate hybridizations), taxa delimitation is often problematic (Liu *et al*. 2009). However, since for our main study objective the taxonomic state of the hybrids was of minor relevance, we pooled the hybrid data for analyses. With more information about morphological, karyological and molecular differentiation of hybrid taxa, future studies should try to elucidate the reproductive isolation vs. intermixture of those distinct types.

Of the species studied, only *G. lutea* is fully fertile (for the study region: Henker 2005; Schnittler *et al*. 2009), the same applies to $6x$ populations of *G. megapolitana* (Henker 2005). The other taxa are, at least regionally in Mecklenburg-Western Pomerania, reported to be totally ($5x$ *G. pratensis*, Mesícek & Hrouda 1974; Henker 2005) or partially sterile (sterile $5x$ but fertile $6x$ *G. pomeranica*, Henker 2005, p. 74). This sexual failure should limit or even prevent genetic interchange in the study region; and primary hybrids of *G. pratensis* and *G. lutea* are so far only assumed for one population from Saxony-Anhalt (John *et al*. 2004; Peterson *et al*. 2004b). However, our results show that this assumption does not hold true: natural seed set, pollen data, and crossing experiments revealed that all taxa and nearly all populations are at least partially fertile (Table 1), although hybrids and *G. pratensis* often exhibited reduced pollen viabilities and reproductive success. The reduced fertility was also indicated by the frequent occurrence of fruits with high numbers of deformed and/or aborted seeds (Table 1).

Both pollen size and viability show consistent differences, with only marginal overlap between *G. pratensis* and *G. lutea* but intermediate values for the hybrids (Fig. 1). Generally, pollen size is positively correlated with ploidy level, though not necessarily in linear fashion (Johansen & von Bothmer 1994; Katsiotis & Forsberg 1995, but see Marciniuk *et al*. 2010). With *G. lutea* being hexaploid, the measurements would indicate a comparable status for hybrid populations hyb1–4 and hyb7 with similar-sized pollen grains (Fig. 1a). This finding agrees

with the count for population hyb4 ($2n = 72 = 6x$, Henker 2005), but hyb1 is reported to correspond roughly to $7x$ (Henker 2005). For populations hyb2–3 and hyb7 no ploidy data are available, but morphological features (e.g. robustness, leaf tips) provided further support for a closer relation to *G. lutea*.

In taxa with anorthoploid levels, meiosis is often highly irregular (Mesícek & Hrouda 1974 for $5x$ *G. pratensis*; Westergård 1936 for $9x$ *G. spathacea*). Mixed chromosomal states due to hybrid origin or very high ploidy levels can have the same effect as they hamper pairing and separation of homologues (see Ramsey & Schemske 2002 for other effects causing polyploid infertility). This can result in pollen grains with different aneuploid and euploid chromosome numbers (Ramsey & Schemske 2002), which might also differ in size (Mesícek & Hrouda 1974) and fertility. Accordingly, we found a higher variance in size measures for *G. pratensis* ($5x$) as well as hybrid pollen grains (cf. 5–$7x$) compared to most *G. lutea* populations (Table 1). Even in the latter species, size differences can occur, but we did not observe the dwarf pollen reported by Mesícek and Hrouda (1974).

The mean viability of *G. lutea* pollen detected in our study ($60.7 \pm 21.2\%$) is lower than 'fertility' values listed by Mesícek and Hrouda (1974; 74.0% + 7.5% stained dwarf microspores). For *G. pratensis*, these authors determined 64.2% in one $5x$ population with 'extremely irregular' meiosis compared to 84.4–95.2% in three $4x$ populations from eastern Slovakia (probably belonging to the distinct species *G. paczoskii* [Zapal.] Grossh.) with 'essentially regular' meiosis (Mesícek & Hrouda 1974, p. 362). This underlines a better viability of pollen from orthoploid plants. Accordingly Gargano *et al*. (2007) detected the highest ratio of normal : deformed pollen grains in $6x$ *G. lutea* compared to other $3x$ and $7x$ taxa. The few available pollen viability figures for the investigated *Gagea* taxa are usually higher than our data (Fig. 1b). Reasons can be natural variation (compare low viability in population lut4; or Zarrei & Zarre 2005 with counts of 93.8% vs. 32.2% for two Iranian *G. lutea* populations), but most likely different methods: most data stem from staining pollen chromatin with carmino acetic acid, whereas the FCR technique relies on the presence of intact plasmalemma and cytosomal esterase activity as proxies for viability. The latter method hence gives lower but more reliable estimates for pollen fertility (compare Trognitz (1991) for correlation of pollen fertility figures derived with various techniques in *Solanum* dihaploids, and Ramsey & Schemske (2002) on pollen viability vs. pollen germinability).

The autogamous crossing experiments revealed that no self-incompatibility (SI) system exists in either of the taxa. The observed reproductive success of selfed plants (measured as percentage of plants with fertile seeds) is often only slightly lower than in allogamous crosses (Fig. 2),

confirming the findings of Nishikawa (1998) for *G. lutea*. As already stated by Graebner and Kirchner (1934) for *G. lutea* and *G. pratensis*, all taxa studied can hence be characterized as facultative autogamous. Within a single flower, allogamy is strongly favored by proterogyny. However, especially geitonogamous autogamy cannot be excluded completely due to the flowering sequence (with flowers in different stages of anthesis on the same plant; see Graebner & Kirchner 1934; Nishikawa 1998 for *G. lutea*) and the possibility of crossings between bulbil progeny from the same genet. In normal situations, most seeds probably result from outcrossing, whereas under less suitable conditions (e.g. closed flowers due to bad weather (Graebner & Kirchner 1934) or missing pollinators) or in monoclonal stands sexual reproduction can be ensured by selfing.

Hybridization in the G. lutea-pratensis *complex*

All interspecific experimental pollinations yielded 'hybridogeneous' seed set (Fig. 2), though with different success with regard to the taxa and direction of pollen flow. Analyses of maternally (cp) vs. biparentally inherited (nuclear) molecular markers indicated multiple primary hybridizations along with further introgressions for hybridogeneous *G. megapolitana* and *G. pomeranica* (Peterson *et al.* 2009). This study presents the experimental proof that backcrosses between hybrid plants and (at least) *G. lutea* as well as crosses between hybrids are possible. By this means, hybrids of higher ranks (and ploidy levels) can be generated. Peterson *et al.* (2009) interpreted the primary fertile 6*x G. megapolitana* as a homoploid between rare 6*x G. pratensis* and 6*x G. lutea* and the nearly sterile 5*x G. pomeranica* as the result of an initial crossing between 4*x G. pratensis* and 6x *G. lutea*. Our data indicate that interbreeding can probably occur between all typical cytotypes (at least 4–6*x* levels; no experimental success but low natural seed set in 7*x* population hyb1), though with better success for ortho- than for anorthoploids and, accordingly, higher fertility of hybrids with even-numbered chromosomal sets.

Due to missing reproductive isolation of the taxa and cytotypes a hybrid swarm is likely to develop if the taxa occur in close proximity (e.g. Hersch-Green & Cronn 2009; Soltis & Soltis 2009), especially in semi-natural conditions (Lamont *et al.* 2003; compare Peterson *et al.* 2009) for anthropogenic influence granting initial contact of *G. pratensis* and *G. lutea*). According to Henker (2005, p. 58) 47% of more than 1000 controlled church yards and cemeteries harbored both *G. lutea* and *G. pratensis*; and mixed populations of hybrids and at least one (often both) parent species are rather common in the study region (our unpubl. data). Hence continuous genetic interchange can be assumed for most of these semi-natural stands.

Generally, the reproductive success in the crossing experiments was rather low, even in *G. lutea*. However, the figures are in the same range as those reported by Nishikawa (1998) for the latter species (see even lower values in Gargano *et al.* 2007). Possible causes for reduced fruit and seed set in experimental compared to natural settings are damage caused by flower manipulations and not fully suitable conditions (e.g. plants were kept bagged to avoid contamination with foreign pollen). Also the crossing data could not be verified statistically due to the limited number of tested samples and populations per cross, and a possible bias due to potential incompatibilities between some combinations. Therefore, proportions in Figure 2 likely underestimate potential seed set in nature.

Nevertheless, the results provide a first general picture of breeding relations of the taxa in the *G. lutea-pratensis* hybrid complex: despite of the assumed anorthoploid state of some hybrid and *G. pratensis* populations, sexual reproduction does occur, though often rarely (Table 1). In the experimental crosses, plants from all hybrid populations but hyb1 produced viable seeds, and nearly all of them also seedlings. Often a medium to significant proportion of normal seeds germinated (Table 2; compare Kondo *et al.* (2004a,b) for germination behavior of *G. lutea*); hence even rare fruiting events might produce a few seedlings per season.

Additionally, even plants with extremely reduced 'maternal fertility' might provide pollen for potential hybridizations (compare data on pollen viability). This additional 'paternal fertility' is best studied for apomictic plant taxa (e.g. in *Taraxacum* spp., Verduijn *et al.* 2004; *Hieracium alpinum*, Mráz *et al.* 2009), but can also be relevant for backcrossed hybrids: Isobe *et al.* (2002) reported a higher pollen than female fertility for backcrossed progeny of *Trifolium medium x T. pratense* hybrids with *T. pratense*, and increasing fertility values for backcrosses of higher rank. This latter phenomenon of increased fertility in higher-rank compared to primary hybrids might account for some differences between hybrid samples and populations encountered in our study.

To verify to what degree normally developed pollen grains are not only viable (as determined in FCR tests) but also euploid, chromosome numbers should be determined. However, even the plants from heptaploid population hyb1, which failed to set seeds in the tested experiments (autogamy, backcross with *G. lutea* pollen), produced some viable pollen grains (Table 1). Also we were able to raise some seedlings from seeds collected in the population. Hence all analyzed hybrid populations are potentially able to (i) reproduce sexually and (ii) interbreed with other hybrid taxa and/or the parent species.

In most years, anthesis of early flowering *G. lutea* and late flowering *G. pratensis* overlap only slightly, thus

reducing the probability of interspecific crosses. Due to this difference in flowering time, proterogyny and the much higher production and better viability of *G. lutea* pollen (Fig. 1b), the formation of hybridogeneous seeds on *G. pratensis* pollinated by *G. lutea* (or the hybrids) is more likely than the reverse combination. In addition, Nishikawa (1998) demonstrated a reduced seed set of the last flowers in an inflorescence of *G. lutea*: Even with artificial pollination the fertilization rate of these flowers was lower than in earlier flowers. Since during anthesis of *G. pratensis* only these last, predominantly male flowers of *G. lutea* are typically still open, this further increases the likelihood that *G. lutea* will act as paternal parent, i.e. pollen donor, in primary hybridizations. Due to the larger overlap in flowering times of the hybrids and *G. lutea*, the latter species might act more regularly as pollen receptor in backcrosses (but see below).

This natural preference of the *G. pratensis x lutea* cross might explain why *G. pratensis* was identified as mother of all hybrid specimens analyzed so far (Peterson *et al.* 2004a, 2008, 2009). However, in our experimental crosses (where we synchronized flowering times) the *G. lutea x pratensis* primary hybridizations were more successful (Fig. 2). This is not in contradiction to the above findings for natural situations, but caused by the partial sterility of the pentaploid *G. pratensis* cytotype found in Mecklenburg-Western Pomerania: Since plants produce much more pollen than ovules, in 5*x* plants chances for viable gametes are much higher if the plant acts as 'father': the numerous pollen grains transferred onto fully fertile *G. lutea* styles in the experiments may occasionally contain euploid grains for successful fertilization, for the few ovules this chance is much lower. In addition to lower seed set, this assumption is aided by the much higher proportion of deformed seeds on *G. pratensis* compared to *G. lutea* plants in these primary hybridizations (Table 2).

The primary hybrid seeds showed a generally reduced viability: seeds from primary hybridizations germinated much less readily (< 30%) than seeds from backcrosses of the hybrids with *G. lutea* (> 60%, Table 2). This might hint on larger developmental problems of primary hybrids due to incompatibilities between the chromosomal sets and/or in aneuploid offspring, which are apparently of less relevance for progeny of 'stabilized' hybrid parents (compare Isobe *et al.* 2002).

The two backcrosses of the hybrids with *G. lutea* yielded a much better success for hybrids than for *G. lutea* as pollen acceptor plants (Fig. 2). In the field this could be related to proterogyny and flowering times (see arguments above for *G. pratensis x lutea*). In our experiments, only the reduced pollen viability of hybrids provides a possible explanation. However, similar findings were reported by Ruhsam *et al.* (2011) for *Geum urbanum x G. rivale* hybrids: F1 hybrids (with only 30% reduced

pollen fertility compared to the parents) failed to form F2 hybrids, but produced first-generation backcrossed progeny with *G. rivale* as pollen parent. Likewise, Wang *et al.* (2001) found a divergent maternal vs. paternal fertility: primary hybrids of *Triticum aestivum x Aegilops cylindrica* can act only as female parent in first-generation backcrosses, and only in later backcrosses is male fertility restored, but remains lower than female fertility (but compare Isobe *et al.* (2002) for better male than female fertility in *Trifolium* hybrid backcrosses).

Conclusion

The taxa of the *Gagea lutea-pratensis* hybrid complex are not yet reproductively isolated but can hybridize and generate hybrids of higher ranks. A stabilization of hybrid types can be expected mainly in isolated, monotypic populations; hybrid swarms with various types are likely to develop in interbreeding hybrid or mixed populations. Especially backcrosses of primary and higher rank hybrids with *G. lutea* are likely, leading to introgression processes. In the study region, the putatively pentaploid *G. pratensis* populations contribute less to interspecific gene flow. Future studies should analyze ploidy levels for all taxa to test to what extent the chromosomal state influences fertility and genetic interchange between taxa.

Acknowledgments

References

Bianchi R. (1946) Untersuchungen über die Fortpflanzungsverhältnisse von *Gagea fistulosa* (Ram.) und *Lloydia serotina*

(Rchb.). *Berichte der Schweizerischen Botanischen Gesellschaft* **56**: 523–583.

Bohdanowicz J. & Lewandowska B. (1999) Participation of endoplasmic reticulum in the unequal distribution of plastids during generative cell formation in *Gagea lutea* (L.) Ker-Gaw. (Liliaceae). *Acta Biologica Cracoviensia Series Botanica* **41**: 177–183.

Chapman M. A. & Burke J. M. (2007) Genetic divergence and hybrid speciation. *Evolution; International Journal of Organic Evolution* **61**: 1773–1780.

Ellstrand N. C., Whitkus R. & Rieseberg L. H. (1996) Distribution of spontaneous plant hybrids. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 5090–5093.

Formella N. (2009) Reproduktionsstrategien bei hybridogenen Arten der Gattung Goldstern (*Gagea*). Diploma Thesis (Biology, Botany), Ernst-Moritz-Arndt-Universität Greifswald, Institut für Botanik und Landschaftsökologie.

Gargano D., Peruzzi L., Caparelli K. F. & Cesca G. (2007) Preliminary observations on the reproductive strategies in five early-flowering species of *Gagea* Salisb. (Liliaceae). *Bocconea* **21**: 349–358.

Graebner P. & Kirchner O. (1934) 11. Gattung *Gagea* Salisb. Goldstern. In: von Kirchner O., Loew E., Schröter C., Wangerin W. & Schröter C. (eds). (Begründer) In *Lebensgeschichte der Blütenpflanzen Mitteleuropas. Spezielle Ökologie der Blütenpflanzen Deutschlands, Österreichs und der Schweiz*. Verlagsbuchhandlung Eugen Ulmer, Stuttgart, pp. 326–355.

Harter D. (2008) Artevolution und Reproduktionsbiologie der Gattung *Gagea* Salisb. (Liliaceae) in Mecklenburg-Vorpommern. Diploma Thesis (Biology, Botany), Freie Universität Berlin.

Hegarty M. J. & Hiscock S. J. (2005) Hybrid speciation in plants: new insights from molecular studies. *The New Phytologist* **165**: 411–423.

Hegarty M. J. & Hiscock S. J. (2008) Review: genomic clues to the evolutionary success of polyploid plants. *Current Biology* **18**: R435–R444.

Henker H. (2005) Goldsterne und Stinsenpflanzen in Mecklenburg-Vorpommern. Teil 1: Die Goldsterne von Mecklenburg-Vorpommern unter besonderer Berücksichtigung kritischer und neuer Sippen. *Botanischer Rundbrief für Mecklenburg-Vorpommern* **39**: 3–90.

Hersch-Green E. I. & Cronn R. (2009) Tangled trios?: characterizing a hybrid zone in *Castilleja* (Orobanchaceae). *American Journal of Botany* **96**: 1519–1531.

Isobe S., Sawai A., Yamaguchi H., Gau M. & Uchiyama K. (2002) Breeding potential of the backcross progenies of a hybrid between *Trifolium medium x T. pratense* to *T. pratense*. *Canadian Journal of Plant Science* **82**: 395–399.

Jakab G. & Molnár V. A. (2011) First record of *Gagea szovitsii* in central Europe. *Biologia* **66**: 433–438.

Johansen B. & von Bothmer R. (1994) Pollen size in *Hordeum* L. – correlation between size, ploidy level, and breeding system. *Sexual Plant Reproduction* **7**: 259–263.

John H., Peterson A. & Peterson J. (2004) Zum taxonomischen Rang zweier kritischer Sippen der Gattung *Gagea* in Mitteleuropa. *Mitteilungen zur floristischen Kartierung in Sachsen-Anhalt* **9**: 15–26.

Katsiotis A. & Forsberg R. A. (1995) Pollen grain-size in four ploidy levels of genus *Avena*. *Euphytica; Netherlands Journal of Plant breeding* **83**: 103–108.

Kearns C. A. & Inouye D. W. (1993) *Techniques for Pollination Biologists*. University Press of Colorado, Niwot, CO, USA.

Kondo T., Miura T., Okubo N., Shimada M., Baskin C. & Baskin J. (2004a) Ecophysiology of deep simple epicotyl morphophysiological dormancy in seeds of *Gagea lutea* (Liliaceae). *Seed Science Research* **14**: 371–378.

Kondo T., Miura T. & Shimada M. (2004b) Effects of storage methods, sowing time, burial depth, and light on the seed germination of *Gagea lutea* Ker-Gawl. *Journal of the Japanese Society of Revegetation Technology* **30**: 546–551.

Lamont B. B., He T., Enright N. J., Krauss S. L. & Miller B. P. (2003) Anthropogenic disturbance promotes hybridization between *Banksia* species by altering their biology. *Journal of Evolutionary Biology* **16**: 551–557.

Levichev I. G. (1999) Zur Morphologie in der Gattung *Gagea* Salisb. (Liliaceae). I. Die unterirdischen Organe. *Flora* **194**: 379–392.

Liu S. C., Lu C. T. & Wang J. C. (2009) Reticulate hybridization of *Alpinia* (Zingiberaceae) in Taiwan. *Journal of Plant Research* **122**: 305–316.

Marciniuk J., Grabowska-Joachimiak A. & Marciniuk P. (2010) Differentiation of the pollen size in five representatives of *Taraxacum* sect. *Palustria*. *Biologia* **65**: 954–957.

Mesícek J. & Hrouda L. (1974) Chromosome numbers in Czechoslovak species of *Gagea* (Liliaceae). *Folia Geobotanica* **9**: 359–368.

Mráz P., Chrtek J. & Šingliarová B. (2009) Geographical parthenogenesis, genome size variation and pollen production in the arctic-alpine species *Hieracium alpinum*. *Botanica Helvetica* **119**: 41–51.

Nishikawa Y. (1998) The function of multiple flowers of a spring ephemeral, *Gagea lutea* (Liliaceae), with reference to blooming order. *Canadian Journal of Botany-Revue Canadienne de Botanique* **76**: 1404–1411.

Pascher A. A. (1904) Übersicht über die Arten der Gattung *Gagea*. *Sitzungsberichte des deutschen naturwissenschaftlich-medizinischen Vereines für Böhmen 'Lotos' in Prag* **52** (N.F. 24): 109–131.

Peakall R., Ebert D., Scott L. J., Meagher P. F. & Offord C. A. (2003) Comparative genetic study confirms exceptionally low genetic variation in the ancient and endangered relictual conifer, *Wollemia nobilis* (Araucariaceae). *Molecular Ecology* **12**: 2331–2343.

Peruzzi L. (2008a) Contribution to the cytotaxonomical knowledge of the genus *Gagea* Salisb. (Liliaceae). III. New karyological data from the central Mediterranean area. *Caryologia* **61**: 92–106.

Peruzzi L. (2008b) Hybridity as a main evolutionary force in the genus *Gagea* Salisb. (Liliaceae). *Plant Biosystems* **142**: 179–184.

Peterson A., Harpke D., Peruzzi L., Levichev I. G., Tison J.-M. & Peterson J. (2009) Hybridization drives speciation in *Gagea* (Liliaceae). *Plant Systematics and Evolution* **278**: 133–148.

Peterson A., Harpke D., Peruzzi L., Tison J. M., John H. & Peterson J. (2010) *Gagea bohemica* (Liliaceae), a highly variable monotypic species within *Gagea* sect. Didymobulbos. *Plant Biosystems* **144**: 308–322.

Peterson A., John H., Koch E. & Peterson J. (2004a) A molecular phylogeny of the genus *Gagea* (Liliaceae) in Germany inferred from non-coding chloroplast and nuclear DNA sequences. *Plant Systematics and Evolution* **245**: 145–162.

Peterson A., John H. & Peterson J. (2004b) Hybridisation in the genus *Gagea* (Liliaceae).—Abstract KV 19-08. *Botanikertagung Braunschweig*, 2004, p. 336.

Peterson A., Levichev I., Peterson J., Harpke D. & Schnittler M. (2011) New insights into the phylogeny and taxonomy of Chinese species of *Gagea* (Liliaceae) – speciation through hybridization. *Organisms Diversity & Evolution* **11**: 387–407.

Peterson A., Levichev I. G. & Peterson J. (2008) Systematics of *Gagea* and *Lloydia* (Liliaceae) and infrageneric classification of *Gagea* based on molecular and morphological data. *Molecular Phylogenetics and Evolution* **46**: 446–465.

Pfeiffer T., Klahr A., Peterson A., Levichev I. G. & Schnittler M. (2012) No sex at all? Extremely low genetic diversity in *Gagea spathacea* (Liliaceae) across Europe. *Flora* 10.1016/j.flora.2012.03.002

R Development Core Team (2011) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna.

Ramsey J. & Schemske D. W. (2002) Neopolyploidy in flowering plants. *Annual Review of Ecology and Systematics* **33**: 589–639.

Rieseberg L. H. & Willis J. H. (2007) Review: plant speciation. *Science* **317**: 910–914.

Ruhsam M., Hollingsworth P. M. & Ennos R. A. (2011) Early evolution in a hybrid swarm between outcrossing and selfing lineages in *Geum*. *Heredity* **107**: 246–255.

Ruthe R. (1893) Eine unbeachtete deutsche Liliacee. *Verhandlungen des Botanischen Vereins der Provinz Brandenburg* **34** (1892; issued 1893): 15–18.

Schnittler M., Pfeiffer T., Harter D. & Hamann A. (2009) Bulbils contra seeds: reproductive investment in two species of *Gagea* (Liliaceae). *Plant Systematics and Evolution* **279**: 29–40.

Slotte T., Huang H. R., Lascoux M. & Ceplitis A. (2008) Polyploid speciation did not confer instant reproductive isolation in *Capsella* (Brassicaceae). *Molecular Biology and Evolution* **25**: 1472–1481.

Soltis D. E., Albert V. A., Leebens-Mack J., Bell C. D., Paterson A. H., Zheng C. F., Sankoff D., dePamphilis C. W., Wall P. K. & Soltis P. S. (2009) Polyploidy and angiosperm diversification. *American Journal of Botany* **96**: 336–348.

Soltis P. S. & Soltis D. E. (2009) The role of hybridization in plant speciation. *Annual Review of Plant Biology* **60**: 561–588.

Trognitz B. R. (1991) Comparison of different pollen viability assays to evaluate pollen fertility of potato dihaploids. *Euphytica* **56**: 143–148.

Verduijn M. H., Van Dijk P. J. & Van Damme J. M. M. (2004) The role of tetraploids in the sexual-asexual cycle in dandelions (*Taraxacum*). *Heredity* **93**: 390–398.

Wang Z. N., Zemetra R. S., Hansen J. & Mallory-Smith C. A. (2001) The fertility of wheat *x* jointed goatgrass hybrid and its backcross progenies. *Weed Science* **49**: 340–345.

Westergård M. (1936) A cytological study of *Gagea spathacea* (with a note on the chromosome number and embryo-sac formation in *Gagea minima*). *Comptes Rendus des Travaux du Laboratoire Carlsberg, Série Physiologique*. **21**: 437–451.

Zarrei M., Wilkin P., Ingrouille M. J., Leitch I. J., Buerki S., Fay M. F. & Chase M. W. (2012) Speciation and evolution in the *Gagea reticulata* species complex (Tulipeae; Liliaceae). *Molecular Phylogenetics and Evolution* **62**: 624–639.

Zarrei M. & Zarre S. (2005) Pollen morphology of the genus *Gagea* (Liliaceae) in Iran. *Flora* **200**: 96–108.