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REGULAR PAPER

Patterns of pollen stainability in polyploids of the genus *Onosma* (Boraginaceae)

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Background and aims – The genus *Onosma* (Boraginaceae) is a taxonomically and phylogenetically very complex plant group, with several taxa of hybrid or polyploid origin. An extensive pollen viability survey within the genus could help to solve some evolutionary or taxonomic incertainties within the genus, but it has never been performed. The aim of the present study is to determine the degree of pollen viability of selected species of various ploidy levels and to investigate if microevolutionary processes, such as polyploidization and hybridization, are associated with variation in pollen viability.
Methods – Pollen staining with lactophenol blue was chosen as an indirect method for determining pollen viability. Ploidy level information was obtained from an extensive literature search. In mixed populations, the ploidy level of individuals was determined based on flow cytometry screening.
Key results – Considerable variation in pollen stainability is present among taxa of *Onosma*. Pollen stainability is high in most diploids (72–98% in mean), allotetraploids (90%) and allohexaploids (97%), but lower in autotetraploids (71%) and allotriploids (2–49%). At the population level, considerable variation in pollen stainability was found in the allotriploid *O. arenaria* (14–83%), while the taxonomically related allotetraploid *O. pseudoarenaria* is less variable (71–99%).
Conclusion – This study shows the value of the staining method in the study of the genus *Onosma*. Low pollen stainability appears to be associated with autopolyploidization as well as recent hybridization processes suggesting the presence of microsporogenesis or microgametogenesis abnormalities. In contrast, pollen stainability is high in stabilized allopolyploids indicating proper meiosis with maintenance of homologous pairing of parental chromosomes and correct pollen development.

Key words – Hybridization, lactophenol blue, male fertility, odd polyploids, *Onosma*, pollen stainability, polyploidization.

INTRODUCTION

Speciation is one of the most interesting phenomena in biology. The origin of a new species can be accompanied and accomplished by several microevolutionary processes which are still not understood satisfactorily (The Marie Curie SPECIATION Network 2011). Some of them, such as the divergence of two geographically distinct units by accumulation of mutations or disruptive selection, are very slow processes, but others, including hybridization and polyploidization, are extremely rapid. These latter two played a major role in the evolution of land plants (Wendel 2000, Mallet 2005), and they are the processes responsible for the formation of homoploids (Rieseberg et al. 1990, Wang et al. 2001, Clay et al. 2012, Peruzzi et al. 2012), autopolyploids (Soltis et al. 2007, Hurka et al. 2012) and allopolyploids (Soltis & Soltis 1993, Soltis et al. 2004, Marhold & Lihová 2006, Clarkson et al. 2010).

To detect polyploidization and hybridization in the modern era of plant systematics and evolutionary research, various sophisticated tools of molecular biology (DNA and isozyme markers or cytogenetic and flow cytometry data) have been successfully applied (Hagerty & Hiscock 2005, Suda et al. 2007, Twyford & Ennos 2012). Such methods are now routinely employed, however, quite expensive and demanding a lot of equipment especially when applying to a rather large set of individuals. In contrast, karyological, morphological, pollen and seed fertility methods are more of a descriptive nature, but in certain plant groups, these techniques might be used in broad surveys to detect potential abnormalities and patterns or to supplement results obtained from other methodological approaches. One of these techniques,

which has been shown useful in many studies investigating polyploidization and hybridization, is the determination of pollen viability. Several studies found correlation between lower pollen viability and meiotic abnormalities in autopolyploids (Solís Neffa & Fernández 2000, Rivero-Guerra 2008, Cohen et al. 2013) or genetic markers distinguishing recent hybrids from their putative parental species (Randell et al. 2004, Lihová et al. 2007). The production of viable pollen is crucial for long-term viability of a lineage as it contributes to successful invasion of unoccupied niches, establishment and survival of stabile populations and further spread of genotypes over distances. Indeed, karyologically variable pollen grains can often lead to the origin of novel polyploid or hybrid cytotypes (Ramsey & Schemske 1998). There are several methods used to determine the degree of viable pollen either indirect through various staining methods or direct through pollen germinability tests (Dafni & Firmage 2000).

The genus *Onosma* L. has undergone multiple polyploidization and hybridization events (Teppner 1988, 2008, Luque 1990, Kolarčik et al. 2010b, 2014). It is the largest genus in Lithospermeae, and one of the largest in Boraginaceae (Cecchi & Selvi 2009, Weigend et al. 2009), with ca. 150 species distributed throughout Anatolia, the Near East and the Mediterranean. Several studies have been conducted on morphological and anatomical features of selected species of *Onosma* (Qureshi & Qaiser 1987, Maggi et al. 2008, Akçin 2009, Binzet & Akçin 2009, Binzet et al. 2010, Akçin et al. 2013, Koyuncu et al. 2013), but few attempts have been made to study the evolutionary history and diversification of the genus, although Teppner gathered karylogical data on the genus (e.g. Teppner 1971, 1980, 1991a, 1991b), and Kolarčik et al. (2010b, 2014), Cecchi et al. (2011) and Mehrabian et al. (2011) conducted molecular studies. The potential of pollen viability data by means of pollen stainability has never been investigated in detail within *Onosma*. Indeed this type of investigation was only shallowly addressed so far (Ranjbar & Almasi 2013, Kolarčik et al. 2014).

In the present study, we were interested in testing if pollen stainability allows for the determination of variable degrees of pollen viability among species of *Onosma* and if these patterns can be attributed to evolutionary processes, such as polyploidization and hybridization. To address this issue, we focus on the estimation of pollen stainability in diploid and polyploid representatives. From the results of previous studies (Teppner 1971, Liu et al. 2004, Madlung et al. 2005, Rivero-Guerra 2008), we expect that polyploid taxa, especially odd allopolyploids, will show decreased pollen stainability due to abortive microgametogenesis or primarily during microsporogenesis, when meiosis takes place.

Our specific questions include: (i) Do the representatives of 'pure' diploid lines produce highly stainable pollen? (ii) Are polyploids (allotriploid, allotetraploid, allohexaploid as well as autotetraploid) characterized by a decreased stainability in comparison with 'pure' diploid species? (iii) Is there a difference in stainability between species with an even and odd level of polyploidization? (iv) Is there any variation in pollen stainability within species across populations? Finally, we were interested in one narrow issue: (v) To investigate in detail pollen stainability of plants in a putatively hybrid population of *O. arenaria* Waldst. & Kit. and *O. fastigiata* (Braun-Blanq.) Lacaita.

MATERIAL AND METHODS

Plant material

Plant material used in this study was sampled exclusively from natural populations. We sampled 12 taxa (66 populations, 1–19 populations per taxon, with 5–166 and 1–31 individuals per species and population, respectively, with a total of 587 individuals, electronic appendix, table 1), all taxonomically determined based on our previous studies (Kolarčik et al. 2010b, 2014) or other published data (Teppner 1996a, 1996b, 2008, Vouillamoz 2001) covering both, diploids and polyploids. To our knowledge, six of the sampled species, *O. echioides* (L.) L., *O. stellulata* Waldst. et Kit., *O. stojanoffii* (Turrill) Teppner, *O. thracica* Vel. (incl. *O. rigida* Ledeb., see Kolarčik et al. 2010b), *O. viridis* (Borb.) Jáv. (incl. *O. tornensis* Jáv., see Mártonfi et al. 2014) and *O. visianii* Clem. are purely diploid, and thus referred to as 'pure' species. *Onosma taurica* Pallas is also included in this group, although some tetraploid individuals have been reported for this species (2n = 4x = 26S, Teppner 1996a). However, the individuals of *O. taurica* investigated here are very probably diploid (Kolarčik et al. 2010b, Kolarčik unpubl. res., table 1). *Onosma fastigiata* includes both diploids and tetraploids. Three studied species were polyploids (table 1). Two taxa *O. arenaria* (in the wide sense, for simplicity hereafter referred to as *O. arenaria*) and *O. pseudoarenaria* Schur (in the wide sense, for simplicity hereafter referred to as *O. pseudoarenaria*) are allotriploid and allotetraploid respectively and the last studied *Onosma malkarmayorum* Teppner is known as allohexaploid. We additionally sampled two populations where hybridization is active. A population in the village of Sutina (Croatia) includes *O. arenaria*, *O. echioides* and the novel well-documented hybrid *O. arenaria* × *O. echioides* (2n = 3x = 6L+15S, Kolarčik et al. 2014, table 1). Another species pair, *O. fastigiata* and *O. arenaria*, co-occurrs in a single known locality in the village of Ceillac (France). Potentially, both species could easily hybridze in sympatry because the chromosome constitution of their pollen grains is likely compatible (Vouillamoz 2001). Since this population has not been critically studied thus far, detailed morphological as well as flow cytometry studies were performed here to precisely identify taxa and cytotypes. Voucher specimens of all material were deposited in the herbarium of the Institute of Botany of the Slovak Academy of Sciences (SAV) (electronic appendix).

Trichome morphology

Trichome morphology was investigated in two species, *O. fastigiata* and *O. arenaria* for the presence of asterosetules on the lower leaf surface which allows distinguishing *O. arenaria* plants from those of *O. fastigiata* (Rübel & Braun-Blanquet 1917). For this analysis, dry leaf parts isolated from all voucher specimens were used. Morphological data was gathered using an Olympus SZ61 stereomicroscope and TESCAN VEGA scanning electron microscope in 'univac' mode, which allows analysis of uncoated samples.

Table 1 – Descriptive statistics of pollen viability data at species level.
Species or entity (in case of identified hybrids) are given (*Taxon*). Ploidy level (*Ploidy*) and number of chromosomes (*Nch*) was inferred based on literature data as indicated by superscripts in *Nch* column: [a] Teppner 1971, Mengoni et al. 2006, Peruzzi & Passalacqua 2008; [b] Teppner 1981; [c] Teppner 1996b; [d] Kolarčik et al. 2010b; [e] Teppner 1991a (datum given for *O. rigida*, see also Material and methods part for explanation), Mártonfiová 2013; [f] Teppner 1991b, Kolarčik et al. 2010b; [g] Teppner 1991b, Mártonfi et al. 2008; [h] Teppner 1971, Vouillamoz 2001, Mártonfi et al. 2008, Kolarčik et al. 2010a; [i] Kolarčik et al. 2014; [j] Vouillamoz 2001; [k] Teppner 1971, Vouillamoz 1999–2000, Vouillamoz 2001, Peruzzi et al. 2004, Mártonfi et al. 2008, Kolarčik et al. 2010a; [l] Teppner 2008. If taxon is of putative hybrid origin, it is indicated in note (*Note*); '?' indicates possible homoploid hybrid origin of *O. echioides* (Kolarčik et al. 2010b). Number of studied individuals and populations (*N/P*) is indicated and minimal (*Min*), mean and standard deviation (*Mean* ± *SD*), maximal (*Max*) and median (*Med*) values of pollen stainability were calculated. [#] L and S denotes particular large and small chromosomes respectively; [*] Pollen stainability was determined for additional 17 and 1 individuals from diploid and tetraploid populations, respectively, for which flow cytometry data were not gathered. These additional estimates fit well to cytotyped ones and did not change *Mean* ± *SD* and *Med* calculates significantly.

Taxon	Ploidy	Nch[#]	Note	N/P	Min	Mean ± SD	Max	Med
O. echioides	2x	14S[a]	homoploid?	114/10	31.8	93.1 ± 11.34	100.0	97.0
O. stellulata	2x	22S[b]	-	9/3	0.0	71.7 ± 40.8	98.0	92.0
O. stojanoffii	2x	14S[c]	-	21/3	63.0	96.0 ± 7.93	100.0	97.0
O. taurica	2x	14S[d]	-	17/2	86.0	97.1 ± 4.62	100.0	99.0
O. thracica	2x	14S[e]	-	35/4	78.0	95.4 ± 5.21	100.0	97.0
O. viridis	2x	14S[f]	-	31/3	86.0	97.2 ± 3.46	100.0	98.0
O. visianii	2x	18S[g]	-	5/3	95.0	98.4 ± 2.07	100.0	99.0
O. arenaria	3x	12L+8S[h]	allopolyploid	61/8	0.0	48.6 ± 31.38	96.0	49.3
O. arenaria × *O. echioides*	3x	6L+15S[i]	allopolyploid	7/1	0.0	1.7 ± 2.21	5.0	0.0
O. fastigiata	2x	12L[j]	-	33/3[*]	0.0	83.1 ± 23.88	99.5	94.4
	3x	18L[j]	autopolyploid	1/1	-	69.3	-	-
	4x	24L[j]	autopolyploid	39/2[*]	0.0	70.9 ± 24.06	100.0	80.8
O. pseudoarenaria	4x	12L+14S[k]	allopolyploid	166/19	0.0	90.4 ± 17.87	100.0	96.4
O. malkarmayorum	6x	38S[l]	allopolyploid	30/5	81.0	97.1 ± 4.83	100.0	99.0

Ploidy level determination

Most data on ploidy level was taken from the literature (table 1). For populations of *O. fastigiata* (5 populations, 7–22 individuals per population, alltogether 73 plants) and one population of *O. arenaria* (18 individuals), we determined ploidy level through the use of flow cytometry. We conducted a two-step flow cytometry procedure that consists of separate nuclei isolation and staining steps (Doležel & Göhde 1995). Samples were prepared with a reference standard (*Solanum lycopersicum* L. cv. Stupnické polní tyčkové rané, 1.96 pg, Doležel et al. 1992) in line with the internal standardization rule (Doležel 1991, Greilhuber et al. 2007). Sample preparation was performed according to Loureiro et al. (2007) with minor modifications. An approximately 2 cm^2 piece of silica-gel-preserved leaves of *Onosma* samples and approximately 0.5 cm^2 piece of fresh leaf material of reference standard were co-chopped using a razor blade in a petri dish containing 1 ml of GPB buffer (Loureiro et al. 2007) to isolate cell nuclei. Suspension was then filtered through a 42 µm nylon filter and supplemented with 2 µl of β-mercaptoethanol, 30 µl RNAse (1 mg ml^{-1}) and 30 µl (1 mg ml^{-1}) of the intercalating stain, propidium iodide. After 10 minutes of incubation at 4°C, the fluorescence intensity of stained nuclei was measured using a Partec CyFlow ML flow cytometer (Partec Gmbh, Münster, Germany), which is housed at the Institute of Biological and Ecological Sciences, P.J. Šafárik University in Košice (Slovakia). One single replicate per sample was made with at least 5,000 measured particles, and the coefficient of variation (CV) was accepted up to 8.00 % for both samples and the reference standard (see e.g. Bardy et al. 2010 for acceptance of similarly high CV values). Each measurement was analysed using FloMax software (ver. 2.7, Partec). The rough estimation of the quantity of DNA per sample was based on the value of the G_0/G_1 peak means according to formula: DNA quantity of sample = DNA quantity of standard × [(G_0/G_1 peak mean of sample)/(G_0/G_1 peak mean of standard)] where G_0/G_1 refers to the population of nuclei in G_0 or G_1 phases of the cell cycle. The DNA quantity was interpreted in light of previously obtained data on karyology and genome size of individuals of *O. fastigiata* and *O. arenaria* from the same populations (Kolarčik et al. 2010a, Kolarčik et al. 2014, Kolarčik et al. unpubl. res.).

Estimation of pollen stainability

Pollen stainability as an indirect method for determining pollen viability was assessed with lactophenol blue staining solution which detects the presence of callose (Kearns & Inouye 1993) and is videly applied (Zonneveld & Van Iren 2001, Kelly et al. 2002, Bleeker & Matthies 2005, Kumar & Singhal 2011). One flower per individual was used as source plant material. In most cases, flowers just prior to anthesis were selected. The entire corolla, with five anthers attached to its inner part, was thoroughly smashed in a drop of distilled water on a microscopic glass slide until pollen grains

were released into a water drop, so the prepared pollen suspension consists of all pollen grains from one flower. Then approximately 30 µl of lactophenol blue staining solution (Fluka analytical) was added to the pollen suspension. After approximately 24 hours of staining incubation, slides were inspected using a Leica DM 2500 microscope equipped with a DFC 290 HD camera and the Leica application suite software (ver. 3.5.0). The percentage of stained pollen grains compared to all inspected pollen grains was calculated in each sample. We usually recorded 100 pollen grains per sample.

Statistical analyses

Our data on pollen viability deviated from normality based on Shapiro-Wilk test (Shapiro & Wilk 1965). We thus arcsin transformed our data prior to further statistical analyses. This, however, did still not lead to a normal distribution of our data. Therefore, to test differences in pollen viability between taxa, ploidies, and populations, we used the Kruskal-Wallis rank sum test (Kruskal & Wallis 1952, nonparametric alternative to analyses of numerical variance) or the Wilcoxon rank sum test (Wilcoxon 1945, nonparametric alternative to t test) in the case of three and more or two tested groups of samples, respectively. To compare three and more groups, the multiple comparison test was conducted after the Kruskal-Wallis rank sum test was performed. Differences in genome size between populations were analysed similarly without a prior data transformation. All statistics were undertaken in R (ver. 2.13.1) (R Development Core Team 2011) using the packages *basic* (R Development Core Team 2011), *stat* (R Development Core Team 2011) and *pgirmess* (Giraudoux 2011).

RESULTS

Trichome morphology

Trichome morphology was investigated in 110 sampled individuals of *O. fastigiata* and *O. arenaria* from the village of Ceillac (France). Ninety-one specimens showed multicellular tubercles without asterosetules, and these were assigned to *O. fastigiata*. The rest of the samples were characterized by the presence of asterosetules on most of the tubercles (some were naked) of the lower leaf surface, and these individuals were assigned to *O. arenaria* (fig. 1).

Ploidy level determination in *O. fastigiata* and *O. arenaria*

Flow cytometry data gathered for 91 individuals (73 of *O. fastigiata* and eighteen of *O. arenaria*) from five populations found 33 diploid (2n = 12L), a single triploid (supposedly 2n = 18L) and 39 tetraploid (2n = 24L) individuals of *O. fastigiata* and eighteen allotriploid (2n = 12L+8S) individuals of *O. arenaria* (fig. 2). The flow cytometry data was in concordance with the morphological data.

Within *O. fastigiata* the five populations were uniform for either diploids or tetraploids, except the population in Col de Alos (ALOS, France) consisting of diploids and a single triploid (fig. 2C). Estimated genome size data are considered preliminary since low accuracy of flow cytometry

Figure 1 – Trichome morphology in *O. fastigiata* (A) and *O. arenaria* (B). Stellate trichomes attached to the base of tubercle in *O. arenaria* allow for clear identification of both species. White arrows indicate trichomes enlarged in insets.

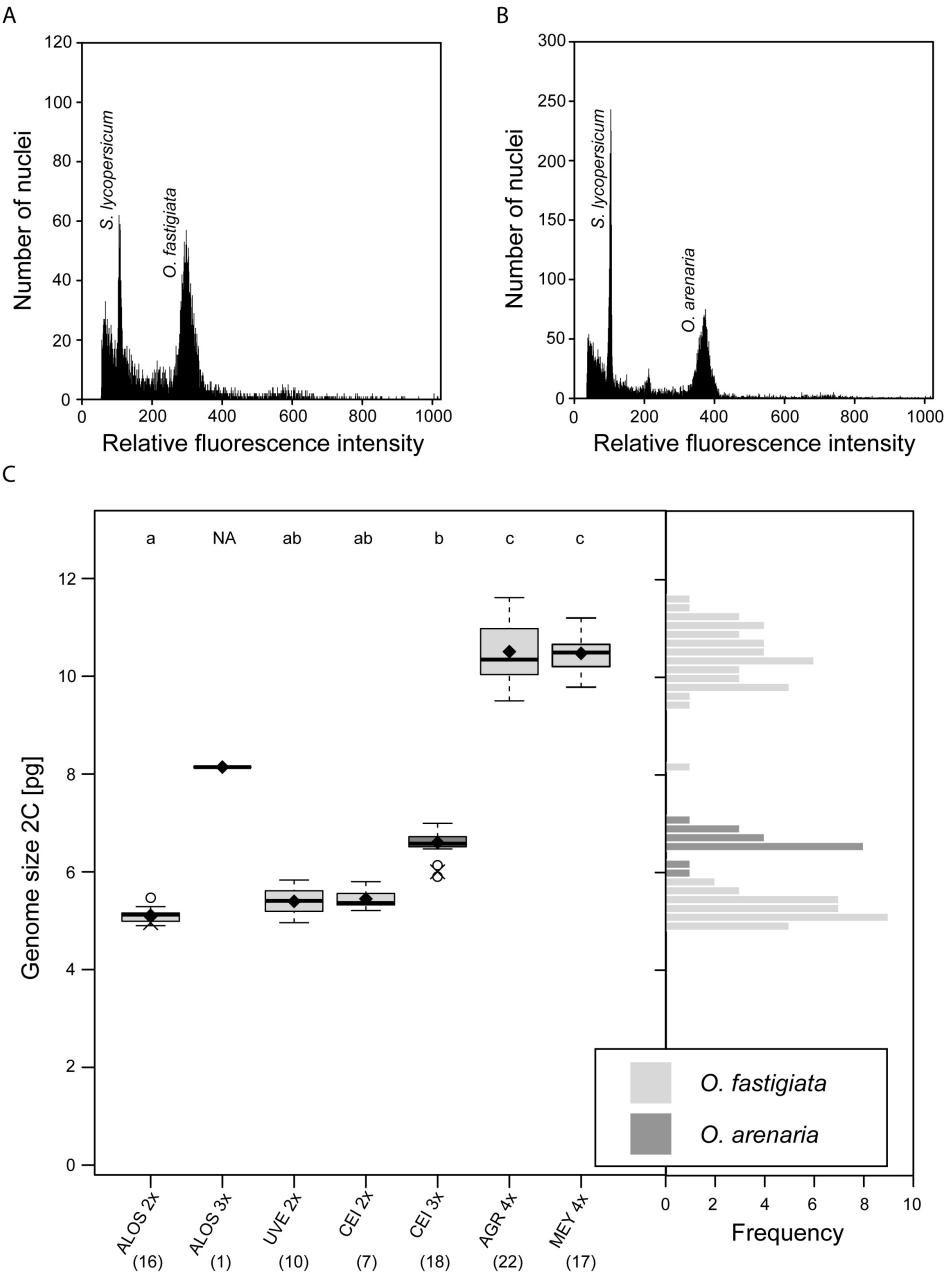

Figure 2 – Representative flow cytometry histograms of *O. fastigiata* (A) and *O. arenaria* (B) and genome size estimations and ploidy level identification in both species (C). A & B, *S. lycopersicum* used as internal reference standard; C, population codes as in electronic appendix. Ploidy level of individuals of *O. fastigiata* – 2x – diploid individuals (2n ~ 12L), 3x – autotriploid individual (2n ~ 18L), 4x – autotetraploid individuals (2n ~ 24L) and *O. arenaria* – 3x – allotriploid individuals (2n ~ 12L+8S). Estimated genome size (Kolarčik et al. unpubl. res.) of the two individuals of *O. fastigiata* ALOSXX4-S6 with 2n = 12L (Kolarčik et al. 2014) and *O. arenaria* CEI3 with 2n = 12L+8S (Kolarčik et al. 2010a) are overimposed (indicated by symbol '×') to allow interpretation of genome size data in terms of ploidy level determination. Numbers in parenthesis indicate sample size. Homogenic groups determined based on multiple comparison test applied after Kruskal-Wallis test are marked by 'a', 'b' and 'c', 'NA' – not applied.

histograms was reached during the measurements (see Material and methods). Genome size estimations, expressed as 2C value, of *O. fastigiata* diploids was 5.27 ± 0.25 pg (mean ± SD), 6.6 ± 0.27 pg for *O. arenaria* allotriploids, 8.14 pg for the *O. fastigiata* triploid (a single individual) and 10.49 ± 0.53 pg for *O. fastigiata* tetraploids. The estimated genome size differed significantly between populations (raw data, $\chi^2 = 78.63$, df = 5, $p < 0.001$; note that the single triploid individual of *O. fastigiata* was not included in the test analysis; see fig. 2).

Pollen grain characteristics

Pollen of 587 individuals was investigated as part of the present study. Pollen grains from 286 individuals were newly examined, and pollen data of another 301 individuals were

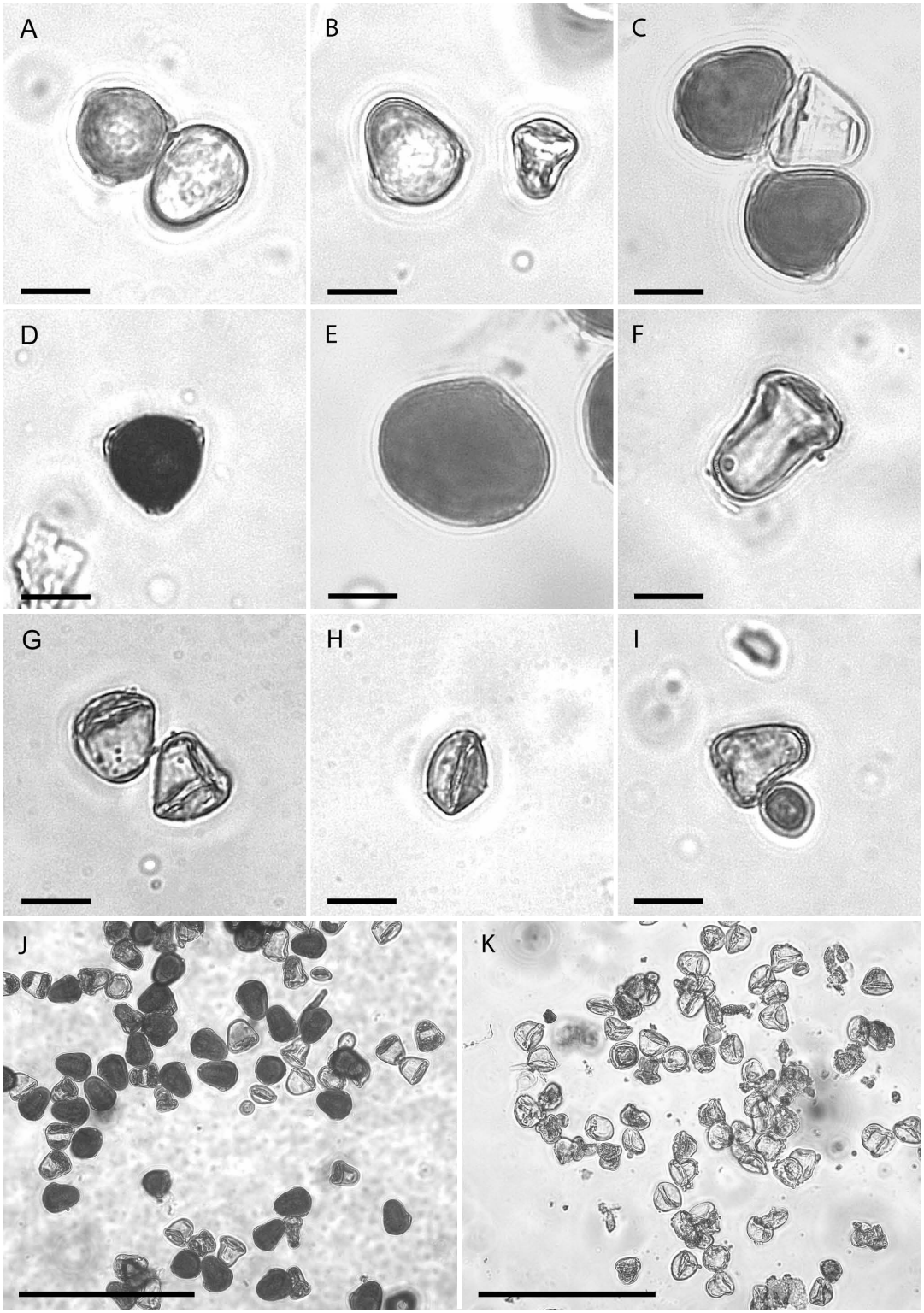

Figure 3 – Pollen grain types found in investigated taxa. A, polar (left) and equatorial (right) view of well-developed pollen grains, without staining; B, well-developed and aborted (shrunken) pollen grains, without staining; C, well-developed (category 1, see text of Results part) and aborted unstained (shrunken, category 3, see text of Results part) pollen grains, after staining; D, equatorial view of well-developed stained pollen grain, after staining; E, rare atypical stained pollen grain of category 4 (see text of Results part), after staining; F, unstained pollen grain of category 2 (see text of Results part), after staining; G–I, various types of aborted unstained (shrunken, category 3, see text of Results part) pollen grains, after staining; J, example of sample with moderate degree of pollen stainability; K, example of sample with low degree of pollen stainability. Scale bars: A–I, 10 µm; J & K, 100 µm. Note: A & B, pollen grains are not stained because no stain was added; C–K, pollen grains are unstained although stain was present.

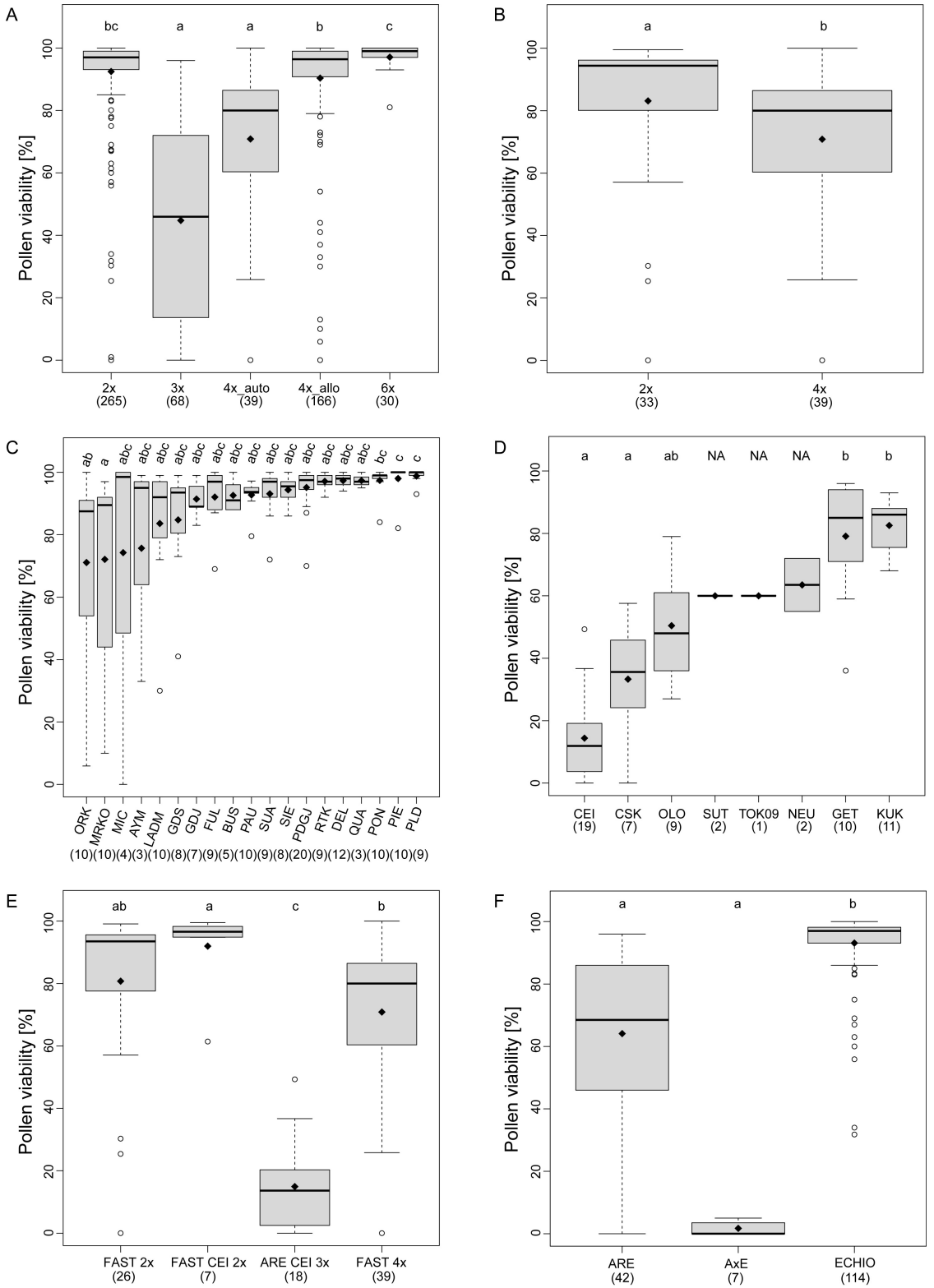

Figure 4 – Comparison of variation in pollen stainability at cytotype and population level. Pollen stainability variation: A, various cytotypes; B, *O. fastigiata* diploids and tetraploids; C, populations of *O. pseudoarenaria*; D, populations of *O. arenaria*; E, *O. fastigiata* ('FAST') and *O. arenaria* ('ARE'), co-occurring individuals in locality Ceillac (France) are indicated by 'CEI'; F, *O. arenaria* ('ARE'), *O. echioides* ('ECHIO') and their hybrid *O. arenaria* × *O. echioides* ('A×E'). 2x – diploid individuals, 3x – triploid individuals, 4x – tetraploid individuals, 4x_auto – autotetraploid individuals, 4x_allo – allotetraploid individuals, 6x – hexaploid individuals. C & D, population codes as in electronic appendix. Numbers in parenthesis indicate sample size. Homogenic groups determined based on multiple comparison test applied after Kruskal-Wallis test are marked by 'a', 'b' and 'c', 'NA' – not applied.

adopted from Kolarčik et al. (2014). Altogether twelve species were analysed (table 1) and more than 68,000 pollen grains were recorded. Usually 100 pollen grains per individual were scored (min-max, 9–353 pollen grains), in only eleven cases (out of 587) fewer than 100 pollen grains were recorded.

All analysed pollen grains can be assigned to four categories (see fig. 3): 1. fully developed stained pollen grains of regular (heteropolar) shape (fig. 3C–D); 2. pollen grains of regular shape but unstained and occassionally smaller than that of category 1 (fig. 3F); 3. various forms of small unstained shrunken pollen grains (fig. 3G–I), 4. very large stained pollen grains of oval shape, which were only found in few samples (fig. 3E). The fourth type of pollen grain was observed only rarely on slides. Only pollen grains of category 1 and 4 were stained and thus considered viable.

Pollen stainability patterns

Pollen stainability was recorded in all 587 individuals of the twelve studied species, and stainability varied significantly between species (arcsin transformed data, $\chi^2 = 218.31$, df = 11, $p < 0.001$, table 1). Highest values were recorded in all 'pure' diploid species (mean 72–98%, median 92–99%, table 1), but also in allotetraploid *O. pseudoarenaria* (mean 90%, median 96%, table 1) and hexaploid *O. malkarmayorum* (mean 97%, median 99%, table 1). The lowest values were found in autotetraploid *O. fastigiata* (mean 71%, median 80%, table 1) and triploid samples (mean 2–49%, median 0–49%, table 1). The recent triploid hybrid *O. arenaria* × *O. echioides* contained almost no stainable pollen. Pollen stainability differed significantly between five identified cytotypes, diploids, triploids, autotetraploids, allotetraploids and allohexaploids [arcsin transformed data, $\chi^2 = 191.57$, df = 4, $p < 0.001$, note that only samples of *O. fastigiata* and *O. arenaria* (population Ceillac), ploidy level of which was estimated were included in analysis], with three homogenic groups recognized (fig. 4A).

In *O. fastigiata*, the lower mean pollen viability of 78% reflects cytotype variability. Diploids had a mean pollen viability of 83% (median of 94%), while tetraploids had significantly lower mean pollen viability, 71% (median of 80%, difference based on arcsin transformed data, W = 968, $p < 0.001$; note that only samples ploidy level of which was estimated were included in analysis, fig. 4B).

In general, low variation in pollen stainability was observed within species and cytotypes, and this was the case across and within populations, with the exception of the included hybrid taxa *O. arenaria* and *O. pseudoarenaria* and the diploid species *O. stellulata*. The later was represented only by three populations (nine individuals), two with high and one with low pollen stainability variation (since two populations were represented each only by two inidividuals, the difference in pollen stainability was not tested). In *O. pseudoarenaria*, pollen stainability differed significantly among nineteen populations (arcsin transformed data, $\chi^2 = 57.38$, df = 18, $p < 0.001$). Fifteen populations reached mean pollen stainability above 80%, and only two samples (out of 139 among the fifteen populations) showed pollen stainability below 50%. But four populations, in Aymaville (AYM, NW Italy), Micoli (MIC, NE Italy), Morakovo (MRKO, Montenegro) and Örkény (ORK, Hungary) were characterized by a pollen stainability below 80%, with seven samples (out of 27 from the four populations) exhibiting less than 50% pollen stainability (fig. 4C).

Highly variable patterns in pollen stainability were observed in the allotriploid *O. arenaria.* Pollen stainability differed significantly between the five populations of *O. arenaria* (arcsin transformed data, $\chi^2 = 41.68$, df = 4, $p < 0.001$). Individuals of *O. arenaria* from the populations in Ceillac (CEI, France) and Csákberény (CSK, Hungary) showed the lowest mean pollen stainability per population (14% and 33% respectively) among the included *O. arenaria* accessions (mean 50–83% in the rest of the populations, fig. 4D).

In the Ceillac population (CEI, France), where *O. arenaria* and *O. fastigiata* co-occur, *O. fastigiata* individuals were characterized by a high level of stainable pollen (mean 92%, median 97%), while *O. arenaria* had a very low level (fig. 4E, see also part devoted to *O. arenaria*). This difference was significant by itself as well as when tested in joint analyses with rest of *O. fastigiata* diploids and *O. fastigiata* tetraploids (arcsin transformed data, $\chi^2 = 45.93$, df = 3, $p < 0.001$; note that only samples whose ploidy level was estimated were included in this analysis).

Comparing the pollen stainability of seven samples of the recently identified hybrid between *O. arenaria* and *O. echioides* (Kolarčik et al. 2014) with that from accessions of the progenitors (from various populations except that of *O. arenaria* from Ceillac) showed a high degree of aborted pollen grains in the hybrids (fig. 4F) but this result was only significant between the recent hybrid and *O. echioides* in a joint analysis of all individuals from all three taxa of *Onosma* (arcsin transformed data, $\chi^2 = 79.92$, df = 2, $p < 0.001$).

DISCUSSION

Pollen stainability pattern in relation to degree of ploidy

Estimation of pollen stainability is commonly used to study male vigour in evolutionary studies (Dafni & Firmage 2000). Male partial or complete sterility in polyploids (as determined through staining methods) reflects abnormalities at microsporogenesis mainly during meiosis, e.g. incorrect chromosome pairing (formation of multivalents), problems to ensure correct chromosome allocation (formation of laggards and bridges) or formation of chromosome segments or micronuclei (e.g. Solís Neffa & Fernández 2000, Madlung et al. 2005, Rivero-Guerra 2008, Diao et al. 2010, Paiva et al. 2012). Therefore, pollen stainability has often been examined in studies of hybridization (e.g. Bleeker & Matthies 2005, Lihová et al. 2007, Bureš et al. 2010, Pfeiffer et al. 2013) and plant breeding programs (e.g. Ferrara et al. 2007, Felismino et al. 2012).

In many studies, pollen viability has been estimated by pollen stainability (Kearns & Inouye 1993). Generally, staining methods suffer from a weakness of only estimating potential viability (Dafni & Firmage 2000) that is expressed in particular stages of pollen development, e.g. meiosis, microgametogenesis, pollen release. This can differ from 'true' pollen viability, which is that the pollen grain can germi-

nate and is capable of penetrating the stigmatic tissues and reaching the ovule. In light of this view, staining methods could overestimate pollen viability since even inviable pollen grains could be stainable (Rodriguez-Riano & Dafni 2000). However, assuming that pollen grains from the same or closely related species might develop and therefore react to staining method in the same way, relative pollen stainability or 'potential viability' can be compared among accessions and thus be a valuable estimator in many cases.

In the present study, pollen stainability analyses were performed in twelve species and five different cytotypes of the genus *Onosma*, including 'pure' diploids, allotriploids, autotetraploids, allotetraploids and allohexaploids. Results show that most of the studied taxa are characterized by highly stained pollen of regular heteropolar shape; however, autotetraploids and triploid taxa (or units) are the exceptions. They have lower pollen stainability compared to their diploid/ancestral counterparts (for phylogeny, see Kolarčik et al. 2014) indicating abortive microsporogenesis or microgametogenesis. Pollen grains of these taxa are usually shrunken and irregular in shape. Rarely, some plants contain stainable large pollen grains of oval shape, which are assumed to be unreduced (2n) pollen grains (see also Teppner 1971 for meiotic studies on *Onosma*).

Diploids *O. echioides*, *O. stellulata*, *O. stojanoffii*, *O. taurica*, *O. thracica*, *O. viridis* and *O. visianii* – Our analyses are in line with previous studies (Bureš et al. 2010) and provide evidence that 'pure' diploid species possess pollen with mean pollen stainability above 90% in most taxa, even in *O. echioides*, which is assumed to be of hybrid origin or at least there has been introgression into the 'pure line' (Kolarčik et al. 2010b). If introgression took place in this case, it did not affect pollen stainability or a selection for male fertility was stabilizing the species during evolutionary times (e.g. Rieseberg et al. 1996, Carney et al. 2000). A 'pure' diploid species that displays an exception in terms of pollen stainability is *O. stellulata* with only 72% pollen stainability. This is due to two exteme individuals of this species that have 0 and 1% of pollen stainability coupled with a low number of samples (N = 9, table 1). A clear explanation does not emerge for this pattern. Indeed, each of those extreme individuals originated from different localities, Grobnik (GRB, Croatia) and Konjic (KONJIC, Bosnia and Hercegovina), and there are other co-occurring species of *Onosma* in each of these localities, *O. visianii* and *O. echioides*, respectively. Potentially, hybridization can explain the observed pattern, but no hybrid between *O. stellulata* and one of these co-occuring species has so far been recorded. Other factors affecting pollen stainability could be associated with the uncommon chromosome composition of the species, which is 2n = 22 chromosomes. *Onosma stellulata* has probably evolved via polyploidization and subsequent diploidization from an ancestor with 2n = 14 chromosomes (x = 7), with *O. mattirolii* Bald. or a closely related species as a hypothesized ancestor (Teppner 1971, 1981). If such a genome with this composition still bears instabilities during meiosis, it suggests an interesting species that should be the subject of rigorous research. Finally, pollen viability of particular individuals could be influenced by some unspecified intrinsic (e.g. genetic, Aarts et al. 1995, Yang et al. 2008) factors as well.

Polyploids *O. pseudoarenaria* and *O. malkarmayorum* – Despite their allopolyploid origins (Teppner 1971, Vouillamoz 2001, Teppner 2008, Kolarčik et al. 2010b, Kolarčik et al. 2014), both *O. pseudoarenaria* and *O. malkarmayorum* have very high pollen stainability. These two species are characterized by even chromosome counts, *O. pseudoarenaria* with 2n = 4x = 12L+14S [large (L) and small (S) chromosomes, Teppner 1971, Vouillamoz 2001, Peruzzi et al. 2004, Mártonfi et al. 2008] and *O. malkarmayorum* with 2n = 6x = 38S (Teppner 2008). High pollen stainability found in both taxa suggest correct microgametogenesis and notably regular microsporogenesis, and we can hypothesize that homologous pairing occurs within groups of different chromosome sets (forming predominantly bivalents).

Autopolyploid *O. fastigiata* – Five populations of *O. fastigiata* were analysed for the presence of diploids and tetraploids. Interestingly, each population had a highly uniform cytotype – either diploid or tetraploid (fig. 2C, for a single exception see results). However, within cytotypes preliminary genome size estimates were highly variable within and among populations, which was likely due to technical issues (see Material and methods part) but real variation can not be excluded and should be verified by further detailed analyses. Tetraploids of this species appear to have originated by autopolyploidy. While a critical study has yet to be undertaken, introgression from other taxa which could suggest an allopolyploid origin was not recorded. Within this species tetraploids have lower pollen stainability than diploids. Similar decreased pollen stainability was found in autotetraploids of *Santolina pectinata* Lag. (Rivero-Guerra 2008), *Turnera* L. (Solís Neffa & Fernández 2000; see this example also for opposite case of high pollen stainability found in autotetraploid taxa) or *Hylocereus* (A. Berger) Britton et Rose (Cohen et al. 2013) and attributed to irregular meiosis.

Triploid *Onosma arenaria* and its natural hybrid *O. arenaria* × *O. echioides* – Both taxa contain allotriploid chromosome sets composed of large (L) and small (S) chromosomes (Kolarčik et al. 2014) and we can hypothesize that the lowest values of mean pollen stainability among the studied individuals found in *O. arenaria* and *O. arenaria* × *O. echioides* reflects improper chromosome pairing in meiosis. Moreover, *O. arenaria* had very high variability of pollen stainability across populations (see discussion below) with surprisingly high mean values up to 83% in some populations. Verification of cytotype composition in those respective populations is needed in order to confirm these rare findings in allotriploids.

Variable pollen stainability pattern across populations within stabilised hybrids

In most populations of *O. pseudoarenaria*, individuals possessed high pollen stainability, which suggests that microsporogenesis functions correctly and that homologous bivalents form from the large (L) and small (S) chromosomes. However, in four out of nineteen studied populations, a higher proportion of individuals with decreased pollen stainability was identified. Two of these four populations (Micoli – MIC, NE Italy and Morakovo – MRKO, Montenegro) are the only ones in our sampling that were geographically close

to another species of *Onosma* each, *O. echioides* (MIC) and *O. stellulata* (MRKO). Hybrids between *O. pseudoarenaria* and these taxa have not been reported previously, thus it remains questionable if decreased pollen stainability in populations MRKO and MIC is caused by recent hybridization (by means of introgression). The reason for the observation in the other two populations where *O. pseudoarenaria* is the only *Onosma* species present, Örkény (ORK, Hungary) and Aymaville (AYM, NW Italy), also remains an open question.

In our study we have detected a significant difference between studied populations of *O. arenaria* that are characterized by either extremely high or extremely low pollen stainability. The observed low pollen stainability in some populations might be explained by previous observations of asymmetric male meiosis in this species (Teppner 1971), where production of a considerable percentage of aborted (with micronuclei or without any nucleus) microspores was reported. However, why there is such a difference among populations is still unclear. In some cases, extreme variability in pollen stainability was attributed to climatic conditions caused by latitudinal variation (Parantainen & Pulkinnen 2002). Our sample is currently too small to test any association between pollen stainability and geographical proximity of studied populations. There is a high geographical distance between pairs of populations with high and low pollen stainability in *O. arenaria*. For example, high pollen stainability values for *O. arenaria* were obtained in populations at Getwing (GET, Switzerland) and Jablonov nad Turňou (KUK, Slovakia), while low values were found from populations in Ceillac (CEI, France) and Csákberény (CSK, Hungary); therefore, we do not seem to observe such a latitudinal correlation.

Recent hybrids coping with shock of genome merging

Both *O. fastigiata* (2n = 12L) and *O. arenaria* (2n = 12L+8S) occur in close sympatry in a single known locality in Ceillac (France), and they are morphologically very similar. In fact, only the close inspection of trichome morphology allows an accurate identification (figs 1 & 2). Our data demonstrate that this *O. arenaria* population is characterized by the lowest mean pollen stainability among all *O. arenaria* populations studied, although not significantly different from most of them. On the other side, *O. fastigiata* individuals from the same locality have very high pollen stainability. *Onosma fastigiata*, which produces 2n = 12L chromosomes, bears pollen with n = 6L. *Onosma arenaria* has asymmetric meiosis, and probably retains only one L chromosome set in its pollen cells (n = 6L, see Teppner 1971, also detailed studies in the model genus *Rosa* – Nybom et al. 2004, Lim et al. 2005, Kovařík et al. 2008). Due to an overlapping flowering period and the close proximity of individuals from both species in the field pollen and chromosome material can potentially be easily exchanged between them (Vouillamoz 2001, Kolarčik personal observations). We hypothesize that gene flow might occur bidirectionally with no change in chromosome number and composition of L and S chromosomes, so that the karyological detection of introgressed individuals is very limited. Homology of the large (L) chromosomes of *O. fastigiata* and those of *O. arenaria* has not been studied so far. Two scenarios can be proposed. (i) In one scenario, these L chromosome sets are only partly homologous (homoeologous), L_a and L_b. Some problems in chromosome pairing could occur in hybrid individuals, with $2n = L_aL_b$ or $2n = L_aL_b+S$, resulting from hybridization between an individual of *O. fastigiata* with $2n = L_aL_a$ and one of *O. arenaria* with $2n = L_bL_b+S$. If this is the case, resulting problems in pollen meiosis should lead to decreased pollen viability (and pollen stainability) in both taxa. High pollen stainability is still present in *O. fastigiata* in the Ceillac population, and this could indicate that only unidirectional gene flow from *O. fastigiata* to *O. arenaria* is possible. In the other scenario, (ii) the large L chromosomes of *O. fastigiata* (2n = LL) are homologous with those of *O. arenaria* (2n = LL+S) and can be introgressed, and one explanation for the large decrease in pollen stainability in *O. arenaria* would be the loss of genes (in means of alleles) responsible for correct asymmetric meiosis, which could be present on L chromosmes of *O. arenaria* (such genetic regulation of pairing of univalent forming chromosomes was proposed in *Rosa*, Werlemark 2003). Still other factors potentially responsible for low pollen stainability in *O. arenaria* from the Ceillac population could be considered, but they remain to be identified yet.

The observed low pollen stainability of *O. arenaria* in Csákberény (CSK, Hungary) could be due to hybridization with *O. visianii*, which occurs in neighbouring sites. Occassional hybrids between *O. arenaria* and *O. visianii* were known from Austria (Teppner 1971). However, we do not assume that this is the case, since we did not observe any evident morphological differences of the studied individuals of *O. arenaria* from the CSK population which could indicate hybridization with *O. visianii*. Further both *O. arenaria* and *O. visianii* can be found close together in locality of Jablonov nad Turňou (KUK, Slovakia), and those *O. arenaria* individuals possess very high pollen stainability (fig. 4D).

Very low pollen stainability (below 2%) was found in a few hybrid individuals of *O. arenaria* × *O. echioides*. The hybrid origin of these plants was revealed recently by a combined approach of molecular markers, karyology and flow cytometry (Kolarčik et al. 2014). The maternal ancestor, *O. arenaria* (LLS genome), belongs to the polyploid hybrid group Heterotricha, and *O. echioides* (SS genome) is a diploid taxon of the Asterotricha group. Differences are noted between the pollen stainability of the ancestors and the hybrid. While both parents possess quite high pollen stainability, their hybrid is almost completely sterile, which reflects a large percentage of aborted microsporogenesis or microgametogenesis. It was hypothesized that the abortion stems from irregular meiosis because of the presence of non-homologous LS_aS_b genome composition in the hybrid or the loss of genes responsible for asymmetric meiosis assurance (Kolarčik et al. 2014).

CONCLUSIONS

The present study clearly demonstrates that pollen viability detected by means of pollen stainability differs among taxa and correlates with certain ploidy levels in *Onosma*. In the present study, all but one diploid taxa are characterized by high pollen stainability, this suggests regular pairing of

chromosomes during meiosis and probably correct microgametogenesis. In contrast, polyploid taxa showed variable patterns in pollen stainability. While even allopolyploids did not differ significantly from diploids, autotetraploid and odd polyploids had decreased values compared to their diploid counterparts or ancestors, and this suggests abortion during microsporogenesis and microgametogenesis. Pollen stainability has thus proven to be an easy to implement and useful method to conduct large surveys on pollen viability yielding new insights into hybridization and polyploidization of taxa thereby contributing to the unraveling of their evolutionary history.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo/supp-data), and consist of summary of plant material of the genus *Onosma* used in the present study.

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