Annals of Botany **116**: 301–312, 2015
doi:10.1093/aob/mcv093, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

When sexual meets apomict: genome size, ploidy level and reproductive mode variation of *Sorbus aria s.l.* and *S. austriaca* (Rosaceae) in Bosnia and Herzegovina

Alma Hajrudinović[1], Sonja Siljak-Yakovlev[2], Spencer C. Brown[3], Fatima Pustahija[1], Mickael Bourge[3], Dalibor Ballian[1] and Faruk Bogunić[1,*]

[1]Faculty of Forestry, University of Sarajevo, Zagrebačka 20, 71000 Sarajevo, Bosnia and Herzegovina, [2]CNRS, Université Paris-Sud, AgroParisTech, UMR 8079, Ecologie, Systématique, Evolution, Bât. 360, 91450 Orsay, France and [3]Pôle de Biologie Cellulaire, Imagif, Centre de Recherche de Gif (FRC3115), CNRS, Saclay Plant Sciences, 91198, Gif-sur-Yvette Cedex, France
** For correspondence. E-mail faruk.bogunic@gmail.com*

Received: 18 December 2014 Returned for revision: 23 February 2015 Accepted: 7 May 2015 Published electronically: 25 June 2015

• **Background and Aims** Allopolyploidy and intraspecific heteroploid crosses are associated, in certain groups, with changes in the mating system. The genus *Sorbus* represents an appropriate model to study the relationships between ploidy and reproductive mode variations. Diploid *S. aria* and tetraploid apomictic *S. austriaca* were screened for ploidy and mating system variations within pure and sympatric populations in order to gain insights into their putative causalities.
• **Methods** Flow cytometry was used to assess genome size and ploidy level among 380 *S. aria s.l.* and *S. austriaca* individuals from Bosnia and Herzegovina, with 303 single-seed flow cytometric seed screenings being performed to identify their mating system. Pollen viability and seed set were also determined.
• **Key Results** Flow cytometry confirmed the presence of di-, tri- and tetraploid cytotype mixtures in mixed-ploidy populations of *S. aria* and *S. austriaca*. No ploidy variation was detected in single-species populations. Diploid *S. aria* mother plants always produced sexually originated seeds, whereas tetraploid *S. austriaca* as well as triploid *S. aria* were obligate apomicts. Tetraploid *S. aria* preserved sexuality in a low portion of plants. A tendency towards a balanced 2m : 1p parental genome contribution to the endosperm was shared by diploids and tetraploids, regardless of their sexual or asexual origin. In contrast, most triploids apparently tolerated endosperm imbalance.
• **Conclusions** Coexistence of apomictic tetraploids and sexual diploids drives the production of novel polyploid cytotypes with predominantly apomictic reproductive modes. The data suggest that processes governing cytotype diversity and mating system variation in *Sorbus* from Bosnia and Herzegovina are probably parallel to those in other diversity hotspots of this genus. The results represent a solid contribution to knowledge of the reproduction of *Sorbus* and will inform future investigations of the molecular and genetic mechanisms involved in triggering and regulating cytotype diversity and alteration of reproductive modes.

Key words: Apomixis, cytotypes, genome size, polyploidy, reproduction mode, sexuality, *Sorbus aria*, *Sorbus austriaca*, Rosaceae.

INTRODUCTION

Hybridization coupled with polyploidization (allopolyploidy) represent determinant occurrences shaping plant diversity (Hegarty and Hiscock, 2008; Soltis and Soltis, 2009). Recurrent formation and recombination of allopolyploid genomes may profoundly recompose genome structure, alter gene expression and induce phenotypic changes (Soltis and Soltis, 2009; Ďurkovič *et al*., 2012). In certain groups, hybridization and polyploidy are associated with changes in the mating system, following a familiar direction from sexuality to asexuality (Ozias-Akins and van Dijk, 2007; Potter *et al*., 2007; Talent and Dickinson, 2007*a*, *b*; Cosendai and Hörandl, 2010; Hojsgaard *et al*., 2014). Polyploidy is related to a breakdown of self-incompatibility, allowing uniparental reproduction and apomixis (Mable, 2004; Hörandl, 2010; Ludwig *et al*., 2013).

Apomixis (asexual seed formation), seen as an evolutionary mechanism, provides a way to preserve and maintain particular hybrid and heterozygous genotypes along with genomes having unbalanced numbers of chromosomes, allowing their long-term propagation and persistence (Hörandl, 2006; Ozias-Akins and van Dijk, 2007). Such uniparental reproduction is seen as having advantages in the colonization of disturbed areas and ecologically unfavourable habitats with respect to outcrossing (Hörandl, 2006; Paun *et al*., 2006). The exact molecular and genetic mechanisms triggering and regulating apomixis are still not fully understood; such research is intriguing from both the developmental and the evolutionary perspective (Hand and Koltunow, 2014).

In the genus *Sorbus*, apomixis is a powerful factor in population structuring and divergence. European members of the genus *Sorbus* include sexual diploid species and numerous

© The Author 2015. Published by Oxford University Press on behalf of the Annals of Botany Company.
All rights reserved. For Permissions, please email: journals.permissions@oup.com

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024

polyploids, predominantly apomictic taxa that are concentrated in three diversity hotspots, i.e. Scandinavia, Britain and south-eastern Europe (Jankun, 1993; Warburg and Kárpáti, 1993; Rich *et al*., 2010; Robertson *et al*., 2010). Prevalence of apomictic polyploids across different parts of the European continent appears to have been initially generated by interspecific hybridization and backcrosses of four diploid sexual species: *S. aria*, *S. aucuparia*, *S. torminalis* and *S. chamaemespilus* (Liljefors, 1953, 1955; Warburg and Kárpáti, 1993). This hypothesis implies that interspecific hybridization events have been linked, at the early stages of divergence of the genus, with unreduced gametes in diploids that produced heteroploid progeny, which subsequently participated in further hybridization events.

Diploid *Sorbus* species are outcrossing and self-incompatible, while polyploids are mostly apomictic, thus at least in part reproductively isolated (facultative apomixis) in relation to their parents. These findings have been achieved by embryological (Liljefors, 1953; Jankun and Kovanda, 1986, 1987, 1988), isozymatic (Proctor *et al*., 1989) and molecular (Nelson-Jones *et al*., 2002; Chester *et al*., 2007; Lepší *et al*., 2008, 2009, 2013; Kamm *et al*., 2009; Robertson *et al*., 2010; Ludwig *et al*., 2013) studies. Early embryological observations provided fundamental information on the type of embryo sac, sexual and asexual pathways of embryo development, pseudogamy, mono- and dispermy of central cell nuclei, as well as disturbed meiosis in hybrids and individuals having odd ploidies. Such investigations have provided an invaluable basis and have been most useful for later interpretation of cytometric data to infer sexual and asexual seed origin along with putative pathways of endosperm formation in different plant groups, including *Crataegus*, which was among the first rosaceous genera studied using cytometric seed screening (Talent and Dickinson, 2007*a*, *b*). Molecular markers, particularly microsatellites, combined with ploidy data have provided plenty of important evolutionary information on *Sorbus*, such as mating system discrimination, parentage identification, complex relationship reconstruction and genetic diversity (Chester *et al*., 2007; Lepší *et al*., 2008, 2009, 2013; Robertson *et al*., 2010; Ludwig *et al*., 2013).

Variations in reproduction mode, an extremely important aspect in *Sorbus* evolution, have not been evaluated by flow cytometry. Flow cytometric seed screening (FCSS) has become a prevailing method of analysing reproduction modes, providing reliability, accuracy and speediness with relatively low costs (Doležel *et al*., 2007 and references therein). Flow cytometric seed screening has been applied in some rosaceous genera [*Amelanchier* (Burgess *et al*., 2014), *Crataegus* (Talent and Dickinson, 2007*a*), *Potentilla* (Dobeš *et al*., 2013), *Rubus* (Šarhanová *et al*., 2012)], shedding new light on their mating systems. Data on embryo and endosperm ploidies allow the discrimination of sexual from asexual seed origins and the inference of ploidies of contributing gametes and pathways of endosperm formation. All such information is of outstanding relevance for *Sorbus*, in which the dynamics of ploidy fluctuation is one of the best indicators of ongoing diversification, especially by comparing ploidy data of progenitors with data of their derived progeny. In contrast to molecular markers, information on ploidy levels does not facilitate distinguishing auto- from allopolyploidy nor resolving the exact parentage of hybrid derivatives.

Diploid *Sorbus* are characterized by having an eight-nucleated *Polygonum*-type embryo sac that follows the most common pattern of sexual reproduction, resulting in a diploid embryo and a triploid endosperm (Jankun and Kovanda, 1986). In *Sorbus* polyploid apomicts, megagametophytes are unreduced due to the bypassing of meiosis (apomeiosis), meaning that the embryo develops from the unreduced egg (parthenogenesis), while the unreduced central cell requires fertilization (pseudogamy) for normal endosperm development (Liljefors, 1953; Jankun and Kovanda, 1986, 1987; Ludwig *et al*., 2013). The successful establishment of apomictic polyploids is highly dependent on their adaptive potential to overcome the problem of endosperm imbalance. For normal endosperm development, flowering plants usually require a balanced contribution of maternal and paternal genomes (2 m : 1p) (Scott, 2007) as a necessary precondition for imprinted gene expression (Haig and Westoby, 1991; Vinkenoog *et al*., 2003). An overdose of either parental genome could result in endosperm failure and finally seed abortion (Scott *et al*., 1998). Due to the bypassing of meiosis, fusion of unreduced central cell polar nuclei results in high ploidies of the maternal genome that contravene the balanced ratio in the endosperm. However, pseudogamous apomicts develop mechanisms such as changes in megagametophyte structure and modifications of fertilization behaviour to cope with deviations from the 2 m : 1p balance (Scott, 2007), successfully yielding normally developed seeds. One of the solutions for achieving endosperm balance in apomicts lies in the availability of one spare sperm, leading to dispermy or fertilization of the central cell's nuclei with both sperms (Talent and Dickinson, 2007*a*; Šarhanová *et al*., 2012; Ludwig *et al*., 2013; Burgess *et al*., 2014). *Sorbus* polyploids show different mating strategies for endosperm formation as a prerequisite for successful apomictic seed production (Ludwig *et al*., 2013). Dispermy of the central cell, as a way to overcome the problem of endosperm imbalance, was documented for tetraploid *Sorbus* species in the embryological study of Jankun and Kovanda (1988). *Sorbus* tetraploids are fully self-compatible and capable of self-pollination while triploid apomicts are self-incompatible and require pollen from other related *Sorbus* taxa (Ludwig *et al*., 2013). Male lineages regularly produce fertile pollen (Rich, 2009); apomicts can therefore exchange genetic material via pollen and consequently transfer apomictic genes to their sexual relatives (Whitton *et al*., 2008; Cosendai and Hörandl, 2010).

A critical point in *Sorbus* divergence is the encounter between sexual diploids and apomictic polyploids, usually resulting in heteroploid offspring due to apomicts' polyploidy. The involvement of newly derived offspring continuously drives, among different related sexual and asexual lineages, a reticulation in future hybridization events that produces novel diversity (Robertson *et al*., 2010). Hence, apomictic polyploids play a double role in the formation of particularly complex patterns of variation within *Sorbus*: they preserve particular variation and initiate interploid crosses.

Classical models of polyploid formation require either unreduced gametes in diploid crosses or the exchange of reduced gametes between diploids and tetraploids (Ramsey and Schemske, 1998). Although triploids are considered to be the main link in the formation of tetraploids via the triploid bridge (Nelson-Jones *et al*., 2002; Robertson *et al*., 2004*a*; Talent and Dickinson, 2007*a*, *b*), recent studies have identified tetraploids as the key link in the formation of *Sorbus* triploids (Ludwig *et al*., 2013).

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024

TABLE 1. *Geographical origin of 12 populations of* Sorbus *mother trees covering four cytotypes and corresponding sample sizes (*N*) for genome size, reproductive mode and pollen stainability*

Site	Origin of sample				Number of samples (*N*)				
	Locality	Longitude (E)	Latitude (N)	Altitude (m)	*S. aria* 2*x*	*S. aria* 3*x*	*S. aria* 4*x*	*S. austriaca*	Total *N* per site
1	Grkarica, Mt Igman	18°17′48″	43°44′21″	1350	8, 35, 7	55, 58, 42	11, 30, 5	31, 42, 26	105, 165, 80
2	Pratača, Mt Igman	18°11′29″	43°45′49″	913	12, 8, 6	–, –, –	–, –, –	–, –, –	12, 8, 6
3	Umoljani, Mt Bjelašnica	18°13′34″	43°39′51″	1300	1, –, 1	9, 1, 7	32, 37, 19	10, 2, 3	52, 40, 30
4	Rujište, Mt Prenj	17°57′05″	43°27′26″	890	6, –, 6	–, –, –	–, –, –	–, –, –	6, –, 6
5	Gradac, Posušje	17°23′33″	43°25′30″	760	25, 6, 6	–, –, –	–, –, –	–, –, –	25, 6, 6
6	Borova glava, Livno	17°07′25″	43°46′52″	1183	39, 13, 15	–, –, –	–, –, –	–, –, –	39, 13, 15
7	Bosiljna, Mt Čvrsnica	17°29′27″	43°30′09″	1319	10, 24, –	14, 12, –	1, 2, –	4, –, –	29, 38, –
8	Blidinje	17°30′26″	43°35′59″	1220	7, –, 1	1, –, –	3, 5, 1	14, –, 3	25, 5, 5
9	Kamenica, Mt Oštrelj	16°24′38″	44°28′30″	1210	12, –, 5	–, –, –	–, –, –	–, –, –	12, –, 5
10	Mt Slovinj	16°57′22″	43°59′10″	1380	41, 13, –	–, –, –	–, –, –	–, –, –	41, 13, –
11	Zavaline, Drvar	16°20′34″	44°20′32″	820	9, –, –	–, –, –	–, –, –	–, –, –	9, –, –
12	Vrba	18°34′06″	43°10′52″	1090	–, –, –	–, –, –	–, –, –	25, 15, 5	25, 15, 5
	Total *N* per cytotype				170, 99, 47	79, 71, 49	47, 74, 25	84, 59, 37	380, 303, 158

Owing to extensive recent work on European *Sorbus* species, genome size, ploidy level and chromosome counts have been determined for most of them (Aldasoro *et al*., 1998; Bailey *et al*., 2008; Lepší *et al*., 2008, 2009, 2013; Siljak-Yakovlev *et al*., 2010; Pellicer *et al*., 2012). In general, the most frequent ploidy levels for *Sorbus* taxa are di-, tri- and tetraploidy, while pentaploids are extremely rare (Bailey *et al*., 2008; Pellicer *et al*., 2012).

A recent study on *Sorbus* in Bosnia and Herzegovina detected a mixture of *S. aria*-like cytotypes in coexistence with *S. austriaca*, representing novel diversity in the Balkans (Hajrudinović, 2012), which motivated a more detailed cytometric study of both species. *Sorbus aria s.l.* (subgenus *Aria*) is an aggregate of related taxa exhibiting a series of ploidy levels: diploid ($2n = 2x = 34$), triploid ($2n = 3x = 51$) and tetraploid ($2n = 4x = 68$) (Bailey *et al*., 2008; Rich *et al*., 2010; Siljak-Yakovlev *et al*., 2010; Pellicer *et al*., 2012). It is the most variable and taxonomically intriguing species aggregate of the genus, being involved in most interspecific hybridization events across Europe (Robertson *et al*., 2010). According to recent taxonomic treatment, *S. aria s.s.* represents a sexual diploid species while morphologically distinctive polyploids are designated as apomictic species (Rich *et al*., 2010). On the other hand, *S. austriaca* (subgenus *Soraria*) is considered to be a stable apomictic allotetraploid species ($2n = 4x = 68$) (Kovanda, 1986); it is relatively widespread, growing in the Balkans, Carpathians and eastern Alps (Warburg and Kárpáti, 1993) and contains genomes of *S. aucuparia* and *S. aria s.l.* (Nelson-Jones *et al*., 2002).

The working hypothesis considered was that the presence of apomicts in sexual populations of *Sorbus* leads to the generation of novel polyploid cytotypes, which deeply impact population structuring via apomixis. In this study we carried out a cytometric survey to assess: (1) the range of genome size and ploidy variation in pure and sympatric populations of the studied species; (2) the increase or decrease in progeny ploidy in relation to the parental generation; (3) the frequency of sexuality in polyploids; and (4) the most frequent endosperm formation pathways among sampled polyploids and their requirements for endosperm balance. The panel comprised seven pure populations of diploid *S. aria*, one of tetraploid *S. austriaca* and four of their sympatric populations. We discuss our findings in the context of evolutionary processes governing *Sorbus* diversification. Also, we advocate that the generation of novel diversity is primarily driven by the interaction of sexual and asexual taxa, an evolutionary model as important for *Sorbus* in the Balkans as for the other diversity hotspots for this genus in Europe.

MATERIALS AND METHODS

Plant material

Flow cytometry was done on leaves of 380 *Sorbus* individuals from 12 sites and 303 seeds of 80 *Sorbus* adult individuals from nine natural sympatric and pure populations encompassing each taxon/cytotype group (Table 1, Fig. 1). The number of sampled individuals ranged from 6 to 105 per population (Table 1, Fig. 1). More individuals were sampled at site 1 ($N = 105$) and site 3 ($N = 52$). The reasons for sampling asymmetry were as follows: sites 1 and 3 were initially the research focus as series of ploidy levels in *S. aria s.l.* had been recorded there for the first time in Bosnia and Herzegovina; our intention was to use certain localities with new *Sorbus* diversity as a criterion to designate specific areas within the High Conservation Value Forest programme in Bosnia and Herzegovina; the convenient geographical proximity of these sites was conducive to repeated sampling when needed. Sampled sites mostly represent mixed stands of *Fagus sylvatica* and *Abies alba* in various stages of degradation (coppices, scrublands) on calcareous or dolomitic rocks. Sites 4 and 8 represent degradation stages of *Pinus heldreichii* forests on dolomitic soils. Site 2 represents a relic thermophilous community of *Quercus petraea* and *Fraxinus ornus* while site 5 belongs to degraded forests of *Quercus pubescens*. Degraded sites are mostly exposed to the south-east, sunny and dry.

We labelled each individual at each site due to the need for repeated sampling in different vegetation periods. Pollen stainability analysis covered only part of the study population since some trees did not flower during the sampling period (Table 1).

For the purpose of this study we treated *S. aria* individuals having different ploidy levels as cytotype groups due to their complicated taxonomy. Vouchers of analysed individuals are

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024

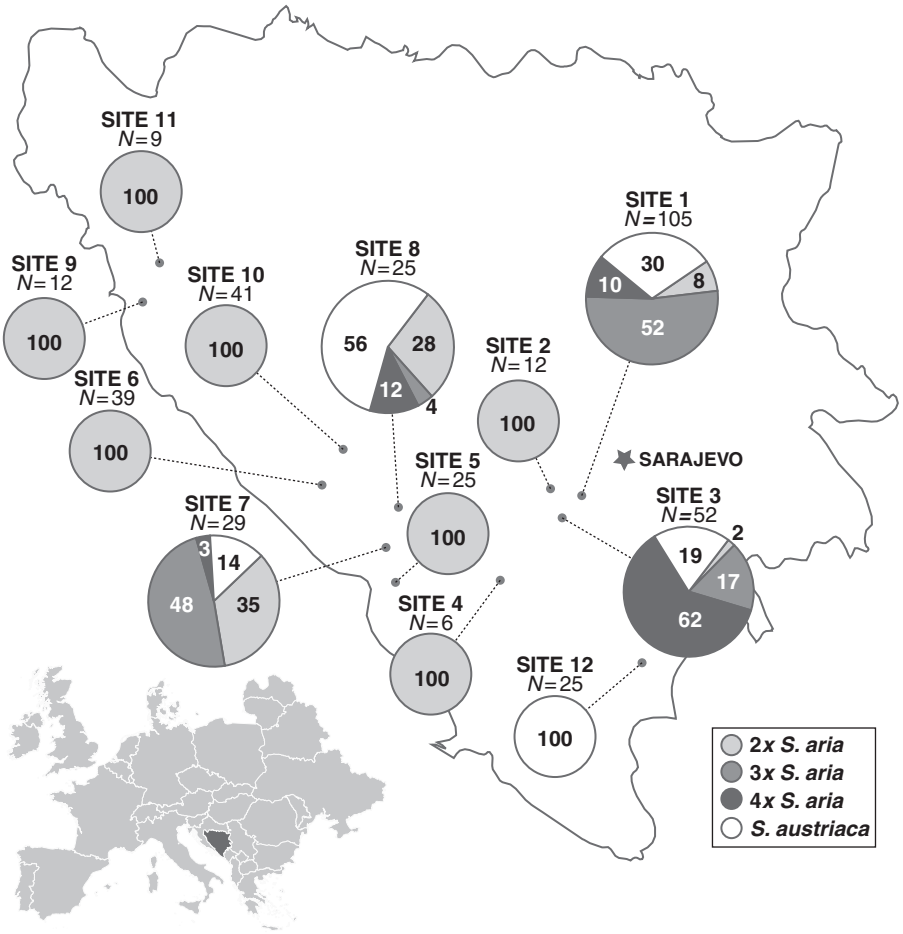

FIG. 1. Geographical distribution of cytotypes and their frequency in each site (% relative to *N* = number of *Sorbus* individuals analysed), for 12 populations in Bosnia and Herzegovina. Site numbers correspond to those in Table 1.

deposited in the Herbarium of the National Museum of Bosnia and Herzegovina (SARA).

Genome size and ploidy level determination

Genome size determination followed the basic protocol described by Marie and Brown (1993). Parts of *Sorbus* fresh leaves (~1 cm^2) were chopped simultaneously with leaf material of internal standard [*Solanum lycopersicum* 'Montfavet 63-5', 2C = 1·99 pg (Lepers-Andrzejewski *et al*., 2011) or *Salvia brachyodon*, 2C = 0·95 pg (Maksimović *et al*., 2007)] using a razor blade in Petri dishes in 600 µL of cold Gif nuclear buffer [45 mM $MgCl_2$, 30 mM sodium citrate, 60 mM 4-morpholinepropane sulphonate pH 7, 0·1 % (w/v) Triton X-100, 1 % polyvinylpyrrolidone (M_r~10 000; Sigma P6755), 5 mM sodium metabisulphite]. The suspension was filtered through a 50-µm nylon mesh (CellTrics, Partec) and RNAse (Roche) was added to 2·5 U mL^{-1}. Nuclei were then stained with propidium iodide (Sigma-Aldrich), with a final concentration of 50 µg mL^{-1}. This preparation was incubated on ice for 15–20 min to obtain full saturation of nuclei with propidium iodide before analysis. The fluorescence of 5000 nuclei was recorded for each sample using a Partec CyFlow SL3 (Partec, Münster, Germany) 532-nm laser cytometer. Fluorescence histograms were analysed using FloMax ver. 2·8 (Partec, Münster, Germany). A linear relationship between the fluorescence signals of the unknown sample and known internal standards was used to calculate the absolute 2C DNA values. Individual DNA ploidy levels (Suda *et al*., 2006) were inferred following earlier chromosome counts on *Sorbus* species and compared with obtained 2C DNA values (Siljak-Yakovlev *et al*., 2010). Each individual was assigned to a particular taxon/cytotype group of di-, tri- and tetraploid *S. aria* and tetraploid *S. austriaca*. Monoploid values of individual cytotypes were calculated using the total 2C DNA value/ploidy level. The mean values of monoploid genome size of cytotype groups were tested using analysis of variance (ANOVA) followed by a *post hoc* Scheffé's test. Prior to ANOVA, the homogeneity of group variances was checked using Levene's test and data distribution was checked using the Kolmogorov–Smirnov test. Analyses were carried out in SPSS ver. 20 (IBM, Armonk, NY, USA).

Flow cytometric seed screen

Flow cytometric seed screening (Matzk *et al*., 2000) was successfully conducted on 303 seeds collected from attached fruit

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024

TABLE 2. *DNA content of* S. aria s.l. *and* S. austriaca *leaf nuclei and deduced ploidy*

Species	DNA ploidy	*N*	2C DNA		CV (%)	1C*x* DNA	
			Mean ± s.d. (pg)	Min–max (pg)		Mean ± s.d. (pg)	Mean (Mbp[a])
S. aria	2*x*	170	1·392 ± 0·034	1·293–1·476	2·4	0·696 ± 0·017**	681
	3*x*	79	2·010 ± 0·071	1·787–2·200	3·5	0·670 ± 0·024	655
	4*x*	47	2·696 ± 0·082	2·513–2·833	3·0	0·674 ± 0·020	659
S. austriaca	4*x*	84	2·722 ± 0·075	2·520–2·944	2·8	0·680 ± 0·019	665
Total *N*		380					

[a]1 pg = 978 Mbp (Doležel *et al*., 2003).
**Significant difference at $P \leq 0{\cdot}01$.

on 65 *S. aria s.l.* and 15 *S. austriaca* mother trees, previously cytotyped (Table 1, Supplementary Data Table S1). Only well-formed seeds, dried for 20–30 h at room temperature and kept in paper bags at 4 °C prior to analysis, were used. Each seed was analysed separately. Entire seeds were co-chopped with part of a fresh leaf (0·5 cm^2) of internal standard *Oryza sativa* ssp. *japonica* 'Nipponbare' [2C = 0·9 pg (Uozu *et al*., 1997)] in cold Gif nuclear buffer. (This internal standard with nuclei below the 2C level of the *Sorbus* spp. has the advantage of avoiding any confusion with the numerous nuclear DNA levels occurring in some seed samples.) Prior to this main analysis, seeds with seed coats removed were also cytotyped as a trial but no differences were observed in fluorescence peaks compared with entire seeds, except in one case in which the seed coat, which was formed from maternal tissue, showed a trace of diploid nuclei while the embryo was triploid (Supplementary Data Fig. S1). The rest of the procedure followed the steps described for genome size and ploidy level determination, but taking both linear and log histograms after the fluorescence had been divided between two photomultipliers with a 50/50 mirror. (Because of the laser beam point focus, the fluorescence signal can be partly clipped on this 532-nm cytometer, so a correction factor must be used to interpolate values for large nuclei, namely 2–10 % depending upon their size. This factor was assessed experimentally by analysing endoreplicated foliar nuclei from *Phalaenopsis* spp., shown to be perfectly 2, 4, 8 and 16C on another cytometer.) Endosperm ploidy was calculated using the inferred monoploid DNA amount of the embryo of the same seed. Estimated DNA ploidies of embryo and endosperm were compared to distinguish between sexual and apomictic origin for each analysed seed and to propose fertilization pathways according to Talent and Dickinson (2007*a*, *b*), Hörandl *et al*. (2008), Cosendai and Hörandl (2010), Šarhanová *et al*. (2012) and Burgess *et al*. (2014), all dealing with plant groups having the same *Polygonum*-type of embryo sac as *Sorbus* (Liljefors, 1953).

Pollen viability

Pollen viability estimation was performed using Alexander's stain (Alexander, 1969). Pollen walls stain green and the cytoplasm red so potentially viable pollen grains can be easily identified. Anthers with fresh pollen grains from different flowers of each analysed individual were gently pressed into a drop of Alexander's stain on a microscopic slide and left with a coverslip overnight at room temperature. One hundred pollen grains were assessed for each of 158 individuals analysed (Table 1).

Seed set

Potential fertility of cytotypes was examined by seed counting. The seeds were collected from the same 80 individuals as for FCSS. Well-formed, firm seeds fully filled with white embryo and endosperm were treated as viable while small, thin and empty seeds, or seeds containing a deformed grey embryo were regarded as sterile. Ten ripe fruits were sampled from each tree. Seeds were extracted and cut in half and the well-formed ones were counted for each fruit and scored as the mean value of an individual. These individual values were used to calculate the mean of well-formed seeds for each taxon/cytotype group.

RESULTS

Nuclear DNA content and ploidy variation

Analysis of nuclear DNA content yielded stable histograms and high-resolution peaks, providing accurate estimation with low coefficients of variation (CVs; mean CV = 3·1 %). Measured on different days, mean CV values among individuals of the same *S. aria* cytotype and *S. austriaca* ranged from 2·4 to 3·9 %. Nuclear DNA content averages ranged from 1·39 pg in diploid *S. aria* to 2·72 pg in tetraploid *S. austriaca* (Table 2). Such DNA content variation was related to the presence of di-, tri- and tetraploid levels in our sample for *S. aria* and only tetraploids for *S. austriaca* (Table 2). The ratios between fluorescence peaks of samples and the internal standards were used to deduce different ploidy levels. The values ranged from 1·29 to 1·48 pg for the diploid *S. aria* group, from 1·79 to 2·20 pg for triploids and from 2·51 to 2·83 pg for tetraploids. *Sorbus austriaca* had values ranging from 2·52 to 2·94 pg. Mean values of monoploid genome size [1C*x* (Greilhuber *et al*., 2005)] ranged from 0·67 to 0·70 pg (Table 2). Diploid *S. aria* had the highest 1C*x* DNA values while triploid *S. aria* had the smallest. ANOVA showed significant differences among the groups for monoploid genome size ($F_{3,376} = 40{\cdot}512$, $P < 0{\cdot}001$). Scheffé's test revealed differences between diploids and all other groups ($P < 0{\cdot}01$), while no differences were found among polyploids (Table 2).

Four out of 12 sampled populations were sympatric (sites 1, 3, 7 and 8) and comprised *S. austriaca* and all three *S. aria* cytotypes (Table 1). The distribution of cytotypes was different at each of these sites (Fig. 1). The more extensively sampled sites 1 and 3 clearly had fewer diploid than polyploid *S. aria* individuals. Triploid *S. aria* was the prevailing cytotype at sites 1 (52 %) and 7 (48 %), tetraploid *S. aria* at site 3 (62 %) and

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024

S. austriaca at site 8 (56 %) (Fig. 1). The other sampled populations were pure species having either diploid *S. aria* or *S. austriaca*.

Embryo and endosperm ploidies and origin of seeds

Here, FCSS was used for detailed analysis of *Sorbus* ploidy variations in relation to mother plants and reproductive modes. Only clearly interpretable results are included in the paper, i.e. results for seeds yielding only one fluorescence peak or having a second peak doubling the first peak (e.g. nuclei in G2 phase or trace endoreduplications of the embryo) were not considered. More than one-third of seeds had minor supernumerary peaks corresponding to doubled nuclear DNA levels mirroring embryo and/or endosperm nuclei. Again, these are trace populations of G2-differentiated or endoreduplicated nuclei. Different ploidies of embryos and endosperms reflected different pathways of seed origin (Table 3). At least two different FCSS profiles were determined in each taxon/cytotype group (Table 3, Fig. 2). The results are coherent with either a reduced or unreduced binucleate central cell being fertilized with either one or two sperm cells.

Embryo and endosperm ploidies and seed origin in diploids All seeds (99) from 21 diploid mothers were of sexual origin (Table 3, Supplementary Data Table S1). Most seeds (88) from all mothers had a regular sexual profile with 2*x* embryos and 3*x* endosperms (Fig. 2A) formed via fertilization of meiotically reduced female gametophytes with haploid ($n = 1x$) sperm cells. Increase in embryo ploidy was observed in 11 seeds having 3*x* embryos and 4*x* endosperms (Table 3, Fig. 2B). These seeds came from three out of four analysed diploid plants from site 1, representing 31 % of the total number of analysed seeds (35; Supplementary Data Table S1). The 3*x* embryos and 4*x* endosperms originated from an interploid cross in which a reduced megagametophyte ($n = 1x$) was fertilized with 2*x* sperm cells, either unreduced ($2n = 2x$) from a diploid male parent or reduced ($n = 2x$) from a tetraploid one.

Embryo and endosperm ploidies and seed origin in triploids Triploid mothers (22) produced seeds (70) of apomictic origin (Table 3, Supplementary Data Table S1). Apomeiotic parthenogenetic embryos were always triploid, while endosperm ploidies ranged from 8*x* to 11*x* (Fig. 2E–H). Endosperm ploidies of 8*x*, 9*x* and 10*x* had almost equal frequencies within this group (Table 3). These ploidies indicate diverse pathways of endosperm formation. Endosperm ploidies of 8*x*, 9*x* and 10*x* were derived from unreduced binucleate central cells ($2n = 3x$) fertilized, respectively, by a single 2*x* sperm cell or two reduced 1*x* sperm cells; a single unreduced 3*x* sperm cell or two with unbalanced chromosome numbers; and a single unreduced 4*x* sperm cell or two reduced 2*x* sperm cells.

In addition, six seeds had 11*x* endosperm, implying a rare case of a trinucleate central cell being fertilized with one 2*x* (*n* or 2*n*) sperm cell or two 1*x* (*n*) sperm cells (Table 3, Fig. 2H).

Exceptionally, one seed had a sexually derived embryo corresponding to 4*x* (Table 3, Fig. 2D), probably due to fertilization of the apomeiotic triploid egg cell with 1*x* sperm. The second peak, corresponding to 8*x*, could represent either endoreduplicated embryo nuclei or nuclei in G2 phase (Fig. 2D).

TABLE 3. *Flow cytometric results and hypothesized pathways of seed formation and mode of reproduction in analysed* Sorbus *taxa/cytotypes*

Mother species	Cytometric results				Hypothesized pathways of seed formation						
	Maternal ploidy*	Embryo mean ± s.d. (pg)	Endosperm mean ± s.d. (pg)	Embryo ploidy	Endosperm ploidy	Number of seeds	Egg ploidy	Polar nuclei ploidy/number	Sperm ploidy	Number of sperm fecundating the endosperm	Endosperm maternal : paternal genome ratio	Endosperm/embryo fluorescence (mean ratio)	Seed origin
S. aria	2*x*	1·39 ± 0·06	2·11 ± 0·12	2*x*	3*x*	88	1*x*	1*x*/2	1*x*	1	2 : 1	1·5	Sexual
	2*x*	2·10 ± 0·11	2·82 ± 0·13	3*x*	4*x*	11**	1*x*	1*x*/2	2*x*	1	1 : 1	1·3	Sexual
	3*x*	2·11 ± 0·10	5·81 ± 0·23	3*x*	8*x*	25	3*x*	3*x*/2	1*x* or 2*x*	2 or 1	3 : 1	2·6	Apomictic
	3*x*	2·11 ± 0·09	6·37 ± 0·40	3*x*	9*x*	18	3*x*	3*x*/2	3*x*	1	2 : 1	2·8	Apomictic
	3*x*	2·12 ± 0·07	7·16 ± 0·19	3*x*	10*x*	21	3*x*	3*x*/2	2*x*	2	1·5 : 1	3·1	Apomictic
	3*x*	2·15 ± 0·17	7·67 ± 0·56	3*x*	11*x*	6	3*x*	3*x*/3	1*x* or 2*x*	2 or 1	4·5 : 1	3·4	Apomictic
	3*x*	2·93 ± 0·13	5·88 ± 0·14	4*x*	–	1**	3*x*	–	1*x*	–	–	–	Sexual
	4*x*	2·84 ± 0·05	4·22 ± 0·03	4*x*	6*x*	3	2*x*	2*x*/2	2*x*	1	2 : 1	1·5	Sexual
	4*x*	2·92 ± 0·23	8·12 ± 0·83	4*x*	11*x*	6	4*x*	4*x*/2	3*x*	1	2·7 : 1	2·7	Apomictic
	4*x*	2·80 ± 0·09	8·40 ± 0·26	4*x*	12*x*	64	4*x*	4*x*/2	2*x*	2	2 : 1	2·8	Apomictic
	4*x*	2·89 ± 0·00	10·16 ± 0·00	4*x*	14*x*	1	4*x*	4*x*/3	2*x*	2	3 : 1	3·5	Apomictic
S. austriaca	4*x*	2·92 ± 0·05	7·17 ± 0·16	4*x*	10*x*	3	4*x*	4*x*/2	1*x* or 2*x*	2 or 1	4 : 1	2·5	Apomictic
	4*x*	2·85 ± 0·13	7·86 ± 0·43	4*x*	11*x*	5	4*x*	4*x*/2	3*x*	1	2·7 : 1	2·6	Apomictic
	4*x*	2·75 ± 0·06	8·31 ± 0·22	4*x*	12*x*	51	4*x*	4*x*/2	2*x*	2	2 : 1	2·8	Apomictic
Total seeds						303							

*Data from cytometric leaf measurements.
**Embryo ploidy was increased compared with the mother's ploidy.

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024

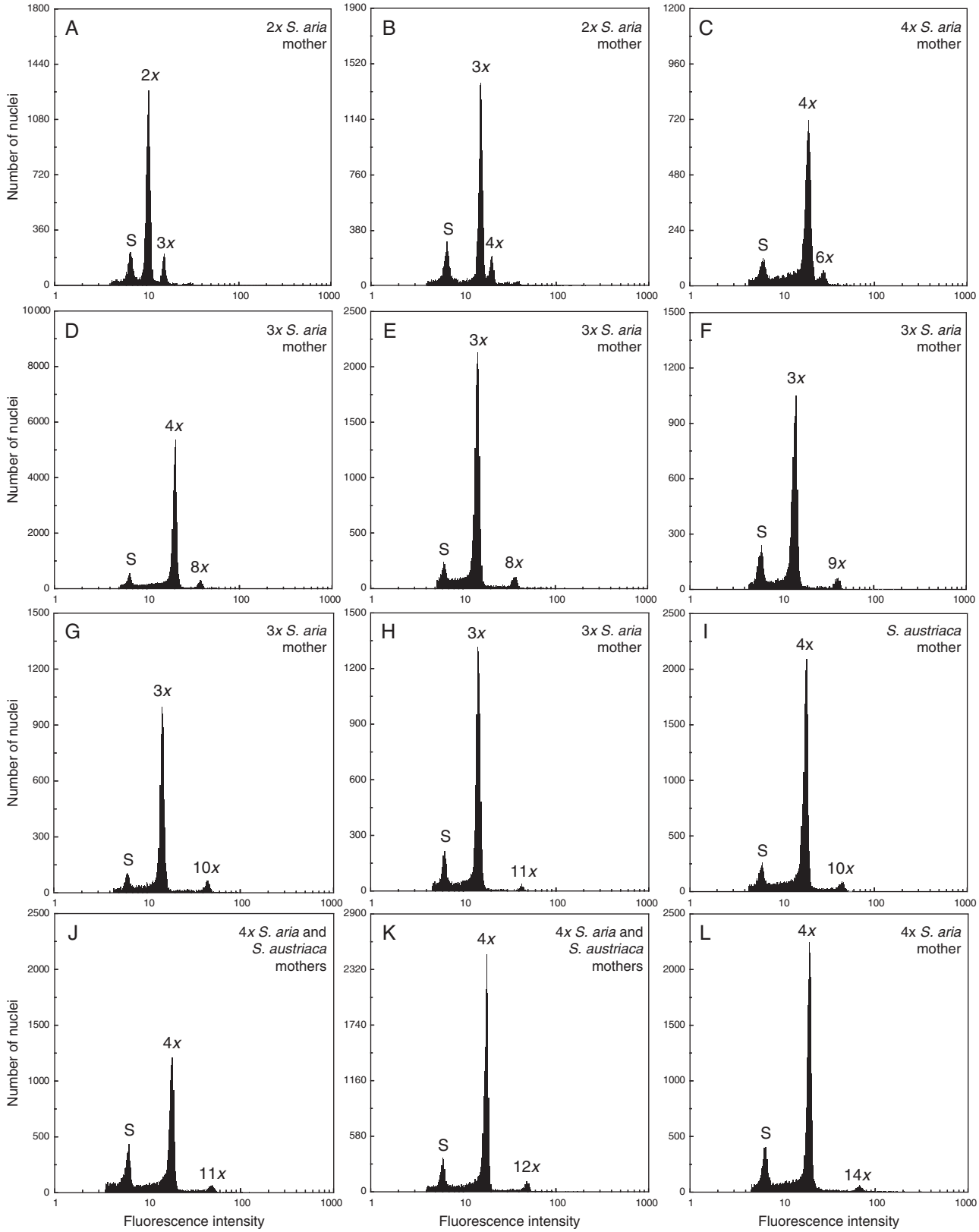

FIG. 2. Flow cytometric seed screen histograms (log abscissa). Histograms of *Sorbus* seeds originated sexually (A–D) and apomictically (E–L). The first fluorescence peak corresponds to the internal standard (S, *Oryza sativa* L. ssp. *japonica* 'Nipponbare'), the second to the embryo and the third to the endosperm.

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024

Embryo and endosperm ploidies and seed origin in tetraploids All seeds (133) of tetraploid mother plants (36) had tetraploid embryo and endosperm ploidies that ranged from 6*x* to 14*x* (Table 3, Fig. 2C, I–L, Supplementary Data Table S1). Sexually generated seeds of *S. aria* had 6*x* endosperms in only three cases (Fig. 2C); they arose via fertilization of meiotically reduced female gametophytes ($n = 2x$) with 2*x* sperm cells. The remaining 130 seeds were formed apomictically (Fig. 2I–L). In 115 apomictically formed seeds in both *S. aria* and *S. austriaca*, the prevailing endosperm ploidy was 12*x*, formed through the fusion of two central cell nuclei ($2n = 4x$) with two 2*x* sperm cells (Fig. 2K). Eleven seeds had 11*x* endosperms (Fig. 2J) suggesting fertilization with 3*x* pollen (a single unreduced sperm cell or two reduced unbalanced sperm cells from triploids). Of the remaining seeds, three had 10*x* endosperms, involving a single reduced ($n = 2x$) sperm cell (Fig. 2I); the last one had 14*x* endosperm, for which the occurrence of an extra central cell nucleus and the fertilization with a reduced ($n = 2x$) sperm cell is proposed (Fig. 2L).

Endosperm balance Balanced endosperm with a maternal-to-paternal genome ratio of 2 m : 1p was present in the majority (74 %) of both sexually and asexually derived seeds (Table 3). Seeds with balanced endosperm had sexual embryo : endosperm profiles of 2*x* : 3*x* (29 %) and 4*x* : 6*x* (1 %) and apomictic profiles of 3*x* : 9*x* (6 %) and 4*x* : 12*x* (38 %). Unbalanced endosperm with a 1 m : 1p ratio was observed in sexually produced seeds from diploid *S. aria* via the involvement of 2*x* male gametes, resulting in a 3*x* : 4*x* profile (4 %) (Table 3). The remaining seeds (22 %) with unbalanced endosperms (1·5 m : 1p, 2·7 m : 1p, 3 m : 1p, 4 m : 1p and 4·5 m : 1p) were of apomictic origin and produced mostly by triploid mothers.

Mean endosperm and embryo ratios were 1·3 and 1·5 in sexually derived seeds and ranged from 2·5 to 3·5 in apomictically derived seeds (Table 3).

Pollen stainability

All analysed groups formed viable pollen. *Sorbus aria* cytotypes showed the highest frequencies of viable pollen (diploids, 83 ± 15 %, range 39–100 %; tetraploids, 86 ± 8 %, range 63–95 %). Mean values for triploid *S. aria* were the lowest (64 ± 16 %, range 32–95 %). *Sorbus austriaca* displayed 74 ± 17 % (range 32–97 %) viable pollen.

Seed set

Individual values for well-formed seeds ranged from 0 to 5 seeds per fruit. Compared with both tri- and tetraploid *S. aria* cytotypes (1·1 ± 0·8; range 0–3), diploid *S. aria* individuals showed almost twice the number of well-formed seeds (2·1 ± 1·0; range 0–5) while *S. austriaca* yielded 1·5 ± 0·9 (range 0–4) well-formed seeds per fruit.

DISCUSSION

Genome size and ploidy variation

In this study, leaf nucleus cytometry precisely differentiated cytotypes. Ploidy levels were easily inferred following monoploid values (1C*x*) for both mother plants and the respective embryos. Monoploid values were quite similar among *S. aria* cytotypes and *S. austriaca* and remained stable across the polyploid series (2*x*, 3*x* and 4*x*). However, the highest difference between mean monoploid values was 0·026 pg, namely between di- and triploid *S. aria* (Table 2). Although minor, differences between monoploid mean values in cytotypes of *S. aria* indicate a genome downsizing trend. The causes of a decrease in monoploid genomes following polyploidization (Leitch and Bennett, 2004) are usually related to the loss of repetitive DNA, such as retroelements or retrotransposons (Bennetzen *et al.*, 2005).

Our estimates of cytotype genome size are similar to those obtained by other authors (Lepší *et al.*, 2008, 2009; Siljak-Yakovlev *et al.*, 2010; Pellicer *et al.*, 2012). The slight differences (~10 %) in relation to other authors for genome size values in diploid *S. aria* and other cytotypes might be attributed to the use of different internal standards, protocols and sample sizes, but also to aneuploidy, small segmental amplifications, deletions, and retrotransposition of DNA short repeats during polyploidization (Bennetzen *et al.*, 2005).

Nevertheless, to date, values for monoploid genomes of all studied *Sorbus* taxa have always shown stability, regardless of hybrid origin and parental combinations or possible autopolyploidy of taxa (Czech Republic: Lepší *et al.*, 2008, 2009; Balkans: Siljak-Yakovlev *et al.*, 2010; Britain: Pellicer *et al.*, 2012; and the present study). This fact argues in favour of limited variation or conservatism of the 1C*x* monoploid genome within the genus. Despite the high rate of hybridization, the numerous combinations of genome crosses and recombination among different taxa across Europe, monoploid genomes remain fairly stable. This scenario requires an evolutionary constraint regulating the stability of monoploid genomes.

Assignment of ploidy level to endosperm was facilitated by using embryo monoploid values, but the endosperm/embryo ratio was sometimes slightly shifted from the expected value. For example, in seeds of diploid mothers the mean ratio was 1·52 ± 0·04 and ranged from 1·41 to 1·64 (Supplementary Data Table S2). Slight deviations of DNA peak positions (from simple multiples of the monoploid value) can also be expected in higher endosperm ploidies (8*x*–14*x*), as the endosperm contains not only maternal genomic DNA but also sperm cells of variable origin, even from different taxa. Thus, the variation might be caused by a series of factors, such as aneuploidy of either central nuclei or sperm cells (variation hardly distinguishable in diploid nuclei but obvious in higher endosperm ploidies) and slight non-linearity of the cytometer (which might induce 2–5 % variation). Nevertheless, the highest endosperm/embryo ratio deviation was detected in a portion of triploid *S. aria* individuals having 3*x* : 9*x* (expected ratio 3, observed ratio 2·84 ± 0·14, CV = 5·0 %) and 3*x* : 11*x* profiles (expected ratio 3·67, observed ratio 3·35 ± 0·15, CV = 4 %) and in *S. austriaca* with a 4*x* : 11*x* (expected ratio 2·75, observed ratio 2·63 ± 0·13, CV = 5·0 %) profile (Supplementary Data Table S2). Higher deviations were observed for odd endosperm ploidies, where values were on the boundary between ploidy levels.

Patterns and processes in single- and mixed-ploidy populations

Our survey of single- and mixed-ploidy *Sorbus* populations reveals the pattern in which ploidy dynamics depend on the interaction of different cytotypes and their mating systems.

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024

In single-cytotype populations of either *S. aria* or *S. austriaca*, no intrapopulational ploidy variation or changes in the mating system were recorded (Fig. 1; Supplementary Data Table S1). On the other hand, mixed-ploidy populations were characterized by different proportions of a particular cytotype, which may reflect active processes within these populations, i.e. one cytotype slowly replacing the other (Sabara *et al*., 2013). In mixed cytotype populations we evidenced ploidy variations in the progeny relative to their maternal plants. Embryo ploidy increases in relation to maternal ploidy levels resulted from interploid crossings in mixed-ploidy *Sorbus* populations. Eleven sexually produced triploid embryos of diploid *S. aria* mothers were fertilized with 2*x* pollen, likely from a tetraploid parent (Table 3). These seeds came from the most extensively sampled population (site 1), where the diploid cytotype was in the minority (eight individuals amongst 105 sampled) and the pressure of polyploid pollen was high (site 1, Table 1, Fig. 1). All analysed *Sorbus* taxa had the same flowering time, and possible 2*x* gamete donors in open pollinations were either tetraploid *S. aria* or *S. austriaca*. Formation of unreduced gametes has been documented in *Sorbus* (Liljefors, 1953; Jankun and Kovanda, 1986, 1987; Aas *et al*., 1994); thus, the involvement of unreduced pollen of diploid *S. aria* cannot be ruled out. However, the presence of unreduced gametes via cases of embryo ploidy increase was not observed in pure populations of diploid *S. aria* (Fig. 1, Supplementary Data Table S1).

The 11 observed triploid embryos represent 31 % of those analysed from diploid mother plants at site 1 (Table 1). This is a high proportion relative to diploid embryos produced; it is reasonable to assume that the number of triploid progeny would be much higher in larger sample sizes of both seeds and maternal trees. These triploid embryos represent novel genetic lineages formed through independent crossing events. Their success and long-term survival will depend critically on potentials determined by their mating system (Hörandl, 2010). Therefore, the genetic structure of triploid populations is not necessarily clonal due to apomixis, but rather comprises multiple clonal lineages having arisen via independent crossing events (A. Hajrudinović, unpubl. res.). The triploid cytotype was prevalent in two out of four sympatric populations analysed (Fig. 1), which indicates their ecological and reproductive success. All triploid mothers produced seeds by apomixis, having the potential to establish themselves in coexistence with other cytotypes. Moreover, they are generally considered to have higher potential for long-distance dispersal and colonization of new habitats compared with their related sexuals (reviewed in Hörandl, 2006).

Ludwig *et al*. (2013) found that apomictic triploid species investigated from Avon Gorge were self-incompatible, requiring pollen of other *Sorbus* taxa for successful endosperm and seed formation. In the majority of cases, triploids used either 1*x* or 2*x* pollen (Table 3). They were also capable of using 3*x* sperm cells, as concluded from the 3*x* : 9*x* embryo : endosperm profile (25 % in the sample of triploid seeds) (Table 3). At all four sympatric sites (Fig. 1), possible pollen donors could be diploid *S. aria*, different genetic lineages of tri- and tetraploid *S. aria*-like and *S. austriaca* individuals producing reduced 1*x*, unbalanced 3*x* and reduced 2*x* sperm cells, but also *S. aucuparia*, which is abundant at these sites (A. Hajrudinović, field observations).

Our findings are concordant with the existing model of apomictic taxon formation, which requires crosses of an apomictic polyploid and sexual diploid progenitor (Robertson *et al*., 2004*a*, 2010; Ludwig *et al*., 2013). We propose that the production of triploids occurs in the direction of pollen exchange from apomictic tetraploids to sexual diploids. This is in favour of triploid formation via the tetraploid bridge (Robertson *et al*., 2004*a*, *b*; Ludwig *et al*., 2013).

Exceptionally, one single case indicating sexuality was detected in a seed with a 4*x* embryo (Table 3, Fig. 2D), which could occur through the union of a 3*x* unreduced female egg cell and a 1*x* sperm. Fertilization of unreduced triploid eggs is a rather rare case but documented in *Sorbus teodori* (Liljefors, 1953) and triploid individuals of *Sorbus chamaemespilus* (Jankun and Kovanda, 1986). The same pathway of origin was established for new tetraploid apomicts in Great Britain: *Sorbus pseudofennica* on Arran (Robertson *et al*., 2004*b*) and *Sorbus* × *houstoniae* in Avon Gorge (Robertson *et al*., 2010). In addition to tetraploidy, these facts also favour a triploid bridge in *Sorbus*.

The exclusive occurrence of polyploid *S. aria* individuals in sympatric populations and their predominantly apomictic reproduction mode (Table 3, Fig. 1) suggest close connections with processes operating elsewhere in Europe in *Sorbus* communities that are ploidy-mixed. Evolutionary processes in *Sorbus* have been thoroughly studied using molecular markers, a prime example being the situation on the island of Arran involving different combinations of species (Robertson *et al*, 2004*a*, *b*). There, initial crossings of diploid sexuals and tetraploid apomicts produced apomictic triploid offspring. Subsequently, unreduced triploid eggs were fertilized by reduced pollen of the parental diploid taxon to produce new tetraploids. Newly derived tetraploids were facultatively apomictic and participated in further crossings to generate new diversity. Our results suggest that the active nature of processes governing *Sorbus* diversity in the Balkans is probably parallel to those determined in Arran and other diversity hotspots of the genus.

While *S. austriaca* produced all seeds via apomixis, several sexually originated seeds were found in *S. aria* tetraploids (Table 3, Fig. 2C). These were documented in two different mothers from site 3. The same mothers also produced seeds of apomictic origin (Supplementary Data Table S1). It is known that tetraploid *Sorbus* apomicts can produce meiotically reduced egg cells and undergo regular sexual pathways (Liljefors, 1953; Jankun and Kovanda, 1986). The preserved sexuality of tetraploids provides an additional evolutionary potential for the exchange of genetic material within *Sorbus* agamic–sexual complexes in Bosnia and Herzegovina. However, ploidy data cannot reveal their exact origins so this question remains open for our *S. aria* polyploids. We assume two different scenarios. The most parsimonious scenario would initially include crossing of apomictic *S. austriaca* reduced 2*x* sperm cells with either reduced 1*x* or unreduced 2*x* megagametophytes of sexual *S. aria*, to produce triploids and tetraploids, respectively. The other involves fertilization of unreduced female gametes of an apomictic triploid to give rise to a tetraploid.

Endosperm formation and balance requirements

The developmental processes and pathways in sexually and asexually formed female gametophytes were thoroughly

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024

presented in embryological and cytological studies of *Sorbus* taxa from Scandinavia and the Czech Republic (Liljefors, 1953; Jankun and Kovanda, 1986, 1987, 1988). In our study, the pathways for seed formation inferred from FCSS data were conclusive and congruent with the body of knowledge derived from embryological studies. FCSS resulted in various embryo : endosperm profiles and pointed to the common pathways of female gametophyte formation and the ploidies of contributing sperm cells (Table 3). Female gametophytes were either reduced or unreduced, with the binucleate central cell fertilized with either one or two sperm cells.

Addition to present knowledge is observed in a tendency towards a balanced 2m : 1p parental genome contribution to the endosperm shared by *Sorbus* diploids and tetraploids, regardless of their sexual or asexual origin (Table 3). The balanced endosperm in seeds of sexual origin was maintained by the exchange of meiotically reduced homoploid gametes. On the other hand, the vast majority of tetraploid apomicts (86 %) maintained a balanced 12*x* endosperm by using either two reduced 2*x* sperm cells or a single unreduced 4*x* sperm cell for the fertilization of the central cell (Table 3). Dispermy of the central cell has been suggested for tetraploids of several rosaceous genera: *Amelanchier* (Burgess *et al*., 2014), *Rubus* (Šarhanová *et al*., 2012) and *Crataegus* (Talent and Dickinson, 2007*a*). Interestingly, the 10*x* endosperm, indicating endosperm imbalance, was prevalent in *Crataegus* and *Rubus* but quite rare in *Amelanchier* and *Sorbus* (Table 3).

The balanced endosperm in triploids' seeds, found in one-quarter of the sample, was achieved by the contribution of three paternal chromosome complements, either by a single 3*x* unreduced sperm cell or two meiotically reduced unbalanced sperm cells, resulting in a 9*x* endosperm as the most balanced state in terms of parental contributions (Table 3). The same uncommon apomictic profile with balanced 9*x* endosperm was detected in *Rubus* triploids (7 %; Šarhanová *et al*., 2012). This pathway greatly depends on the frequency of production of such gametes. Irregular microsporogenesis, resulting in a series of different chromosome numbers (17–25) was reported in triploid *Sorbus bohemica* that had a low pollen stainability of ∼20 % (Jankun and Kovanda, 1987). In our study, all cytotype groups showed high pollen stainability, including triploids, in which stainability reached 95 % in certain individuals (see the Results section).

Most seeds (75 %) from triploid mothers showed deviations from a balanced endosperm, having ratios of 1·5m : 1p, 3m : 1p and 4·5m : 1p (Table 3), as is also frequent in rosaceous triploid apomicts of *Amelanchier* (1·5m : 1p and 3m : 1p, Burgess *et al*., 2014), *Rubus* (1·5m : 1p and 3m : 1p; Šarhanová *et al*., 2012) and *Crataegus* (1·5m : 1p; Talent and Dickinson, 2007*a*), but also *Sorbus* (3 m :1p; Jankun and Kovanda, 1987).

However, tolerance of imbalance and viability in these seeds remains questionable. The analysis of seed set showed that triploids yielded the lowest number of seeds relative to diploids and tetraploids. However, triploids apparently tolerate endosperm imbalances, given their numerical dominance in some of the populations investigated here.

Tolerance of endosperm imbalance potentiates the formation of polyploids via interploidal crosses in the genus (Hörandl *et al*., 2008). This potential particularly refers to unbalanced endosperm with the 1m : 1p ratio observed in sexually produced seeds from diploid *S. aria* via the involvement of 2*x* male gametes, resulting in a 3*x* : 4*x* profile (Table 3). Crossings between diploids and tetraploids probably do occur recurrently, since this was the only observed pathway of triploid formation in the present dataset. In any case, the endosperm balance requirement must always be violated in seeds originating via diploid–tetraploid crosses, regardless of the direction of the cross. Hence, as has been suggested by Ludwig *et al*. (2013), we advocate that formation of *Sorbus* triploids is facilitated by relaxed sensitivity to the endosperm balance requirement.

Concluding remarks

Results on flow cytometry proved valuable in the analysis of ploidy dynamics and the identification of mating systems in *Sorbus*. Cytotype diversity in mixed-ploidy populations demonstrated a link with the coexistence of sexual *S. aria* diploids with apomictic *S. austriaca* tetraploids. Tetraploid-to-diploid gene flow was suggested by combining ploidy data of progenitors and progeny. The consequence of being a *Sorbus* polyploid is a change in reproduction mode that favours apomixis. Triploids exhibited near obligate apomixis, as well as did tetraploid *S. austriaca*. Tetraploids of *S. aria* were characterized by facultative apomixis, having a small portion of retained sexuality. Facultative apomixis provides additional evolutionary potential in further interactions within Bosnian *Sorbus* agamic–sexual complexes. The ecological and reproductive success of triploids and their putative capability for tolerating endosperm imbalance pose intriguing evolutionary questions from developmental perspectives.

Our data show similarities with processes governing *Sorbus* diversity in other hotspots of the genus in Europe. Bosnian *Sorbus* sexual–agamic complexes harbouring mixtures of cytotypes and the interacting sexual and asexual lineages represent potential reservoirs of new biodiversity that merit a status of special concern for conservation. By using cytometric data as a solid baseline, our forthcoming research will use molecular methods to identify precise parentages and the underlying genetic mechanisms.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals and consist of the following. Table S1: flow cytometric seed screening results for 303 *Sorbus aria s.l.* and *S. austriaca* seeds from natural populations in Bosnia and Herzegovina. Table S2: group statistics for observed FCSS profiles in *Sorbus aria s.l.* and *S. austriaca*. Figure S1: DNA histograms of *Sorbus aria* diploid mother tree seeds for FCSS, using linear and log axes.

ACKNOWLEDGEMENTS

We thank two anonymous reviewers for helpful comments on an earlier version of the manuscript. We thank Ante Begić for help collecting the samples in the field. This work was supported by grants from the Environmental Fund of the Federation of BiH (01-02-1053/2012) and the Federal Ministry of Education and Science (05-39-3667-1/14) to F.B.

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024

We kindly acknowledge all supporting facilities from the IBiSA Cytometry Platform of the Imagif Cell Biology Unit at Gif campus (www.imagif.cnrs.fr), including Saclay Plant Sciences (ANR-10-LABX-0040-SPS) and France-BioImaging (ANR-10-INSB-04-01). This article collates results from the ongoing PhD study of A.H.

LITERATURE CITED

Aas G, Maier J, Metzeger S. 1994. Morphology, isozyme variation, cytology and reproduction of hybrids between *Sorbus aria* (L.) Crantz and *S. torminalis* (L.) Crantz. *Botanica Helvetica* **104**: 195–214.

Aldasoro JJ, Aedo C, Navarro C, Garmendia FM. 1998. The genus *Sorbus* (Maloideae, Rosaceae) in Europe and in North Africa: morphological analysis and systematics. *Systematic Botany* **23**: 189–212.

Alexander MP. 1969. Differential staining of aborted and nonaborted pollen. *Stain Technology* **44**: 117–122.

Bailey JP, Kay QON, McAllister H, Rich TCG. 2008. Chromosome numbers in *Sorbus* L. (Rosaceae) in the British Isles. *Watsonia* **27**: 69–72.

Bennetzen JL, Ma J, Devos KM. 2005. Mechanisms of recent genome size variation in flowering plants. *Annals of Botany* **95**: 127–132.

Burgess MB, Cushman KR, Doucette ET, Talent N, Frye CT, Campbell CS. 2014. Effects of apomixis and polyploidy on diversification and geographic distribution in *Amelanchier* (Rosaceae). *American Journal of Botany* **101**: 1375–1387.

Chester M, Cowan RS, Fay MF, Rich TCG. 2007. Parentage of endemic *Sorbus* L. (Rosaceae) species in the British Isles: evidence from plastid DNA. *Botanical Journal of the Linnean Society* **154**: 291–304.

Cosendai AC, Hörandl E. 2010. Cytotype stability, facultative apomixis and geographical parthenogenesis in *Ranunculus kuepferi* (Ranunculaceae). *Annals of Botany* **105**: 457–470.

Dobeš C, Lückl A, Hülber K, Paule J. 2013. Prospects and limits of the flow cytometric seed screen – insights from *Potentilla sensu lato* (Potentilleae, Rosaceae). *New Phytologist* **198**: 605–616.

Doležel J, Bartoš J, Voglmayr H, Greilhuber J. 2003. Nuclear DNA content and genome size of trout and human. *Cytometry* **51**: 127–128.

Doležel J, Greilhuber J, Suda J. 2007. *Flow cytometry with plant cells: analysis of genes, chromosomes and genomes*. Weinheim: Wiley-VCH.

Ďurkovič J, Kardošová M, Čaňová I, *et al.* 2012. Leaf traits in parental and hybrid species of *Sorbus* (Rosaceae). *American Journal of Botany* **99**: 1489–1500.

Greilhuber J, Doležel J, Lŷsak M, Bennett MD. 2005. The origin, evolution and proposed stabilization of the terms 'genome size' and 'C-value' to describe nuclear DNA contents. *Annals of Botany* **95**: 255–260.

Haig D, Westoby M. 1991. Genomic imprinting in endosperm: its effects on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixis. *Philosophical Transactions of the Royal Society, Series B* **333**: 1–13.

Hajrudinović A. 2012. Genome size and morphological diversity of *Sorbus aria* and *S. austriaca* in certain populations from Bosnia and Herzegovina. MSc Thesis, University of Sarajevo, Bosnia and Herzegovina.

Hand ML, Koltunow AM. 2014. The genetic control of apomixis: asexual seed formation. *Genetics* **197**: 441–450.

Hegarty MJ, Hiscock SJ. 2008. Genomic clues to the evolutionary success of polyploid plants. *Current Biology* **18**: 435–444.

Hojsgaard D, Greilhuber J, Pellino M, Paun O, Sharbel TF, Hörandl E. 2014. Emergence of apospory and bypass of meiosis via apomixis after sexual hybridisation and polyploidisation. *New Phytologist* **204**: 1000–1012.

Hörandl E. 2006. The complex causality of geographical parthenogenesis. *New Phytologist* **171**: 525–538.

Hörandl E. 2010. The evolution of self-fertility in apomictic plants. *Sexual Plant Reproduction* **23**: 78–86.

Hörandl E, Cosendai A-C, Temsch EM. 2008. Understanding the geographic distributions of apomictic plants: a case for a pluralistic approach. *Plant Ecology & Diversity* **1**: 309–320.

Jankun A. 1993. Evolutive significance of apomixis in the genus *Sorbus* (Rosaceae). *Fragmenta Floristica et Geobotanica* **38**: 627–686.

Jankun A, Kovanda M. 1986. Apomixis in *Sorbus sudetica* (Embryological studies in *Sorbus* 1). *Preslia* **58**: 7–19.

Jankun A, Kovanda M. 1987. Apomixis and origin of *Sorbus bohemica* (Embryological studies in *Sorbus* 2). *Preslia* **59**: 97–116.

Jankun A, Kovanda M. 1988. Apomixis at the diploid level in *Sorbus eximia* (Embryological studies in *Sorbus* 3). *Preslia* **60**: 193–213.

Kamm U, Rotach P, Gugerli F, Siroky M, Edwards P, Holderegger R. 2009. Frequent long-distance gene flow in a rare temperate forest tree (*Sorbus domestica*) at the landscape scale. *Heredity* **103**: 476–482.

Kovanda M. 1986. *Sorbus scepusiensis*, a new species of *Sorbus* (Rosaceae) from Eastern Slovakia. *Willdenowia* **16**: 117–119.

Leitch IJ, Bennett MD. 2004. Genome downsizing in polyploid plants. *Biological Journal of the Linnean Society* **82**: 651–663.

Lepers-Andrzejewski S, Siljak-Yakovlev S, Brown SC, Wong M, Dron M. 2011. Diversity and dynamics of plant genome size: an example of polysomaty from a cytogenetic study of Tahitian vanilla (*Vanilla ×tahitensis*, Orchidaceae). *American Journal of Botany* **98**: 986–997.

Lepší M, Vít P, Lepší P, Boublik K, Suda J. 2008. *Sorbus milensis*, a new hybridogenous species from northwestern Bohemia. *Preslia* **80**: 229–244.

Lepší M, Vít P, Lepší P, Boublik K, Kolář F. 2009. *Sorbus portae-bohemicae* and *Sorbus albensis*, two new apomictic endemic species recognized based on revision of *Sorbus bohemica*. *Preslia* **81**: 63–89.

Lepší M, Lepší P, Sádlo J, Koutecký P, Vít P, Petřík P. 2013. *Sorbus pauca* species nova, the first endemic species of the *Sorbus hybrida* group for the Czech Republic. *Preslia* **85**: 63–80.

Liljefors A. 1953. Studies on propagation, embryology and pollination in *Sorbus*. *Acta Horti Bergiani* **16**: 277–329.

Liljefors A. 1955. Cytological studies in *Sorbus*. *Acta Horti Bergiani* **17**: 47–113.

Ludwig S, Robertson A, Rich TCG, *et al.* 2013. Breeding systems, hybridization and continuing evolution in Avon Gorge *Sorbus*. *Annals of Botany* **111**: 563–575.

Mable BK. 2004. Polyploidy and self-compatibility: is there an association? *New Phytologist* **162**: 803–811.

Maksimović M, Vidic D, Miloš M, Šolić ME, Abadžić S, Siljak-Yakovlev S. 2007. Effect of the environmental conditions on essential oil profile in two Dinaric *Salvia* species: *S. brachyodon* Vandas and *S. officinalis* L. *Biochemical Systematics and Ecology* **35**: 473–478.

Marie D, Brown SC. 1993. A cytometric exercise in plant DNA histograms, with 2C values for 70 species. *Biology of the Cell* **78**: 41–51.

Matzk F, Meister A, Schubert I. 2000. An efficient screen for reproductive pathways using mature seeds of monocots and dicots. *Plant Journal* **21**: 97–108.

Nelson-Jones EB, Briggs D, Smith AG. 2002. The origin of intermediate species of the genus *Sorbus*. *Theoretical and Applied Genetics* **105**: 953–963.

Ozias-Akins P, van Dijk PJ. 2007. Mendelian genetics of apomixis in plants. *Annual Review of Genetics* **41**: 509–537.

Paun O, Stuessy TF, Hörandl E. 2006. The role of hybridization, polyploidization and glaciation in the origin and evolution of the apomictic *Ranunculus cassubicus* complex. *New Phytologist* **171**: 223–236.

Pellicer J, Clermont S, Houston L, Rich TCG, Fay MF. 2012. Cytotype diversity in the *Sorbus* complex (Rosaceae) in Britain: sorting out the puzzle. *Annals of Botany* **110**: 1185–1193.

Potter D, Eriksson T, Evans RC, *et al.* 2007. Phylogeny and classification of Rosaceae. *Plant Systematics and Evolution* **266**: 5–43.

Proctor MCF, Proctor ME, Groenhof AC. 1989. Evidence from peroxidase polymorphism on the taxonomy and reproduction of some *Sorbus* populations in south-west England. *New Phytologist* **112**: 569–575.

Ramsey J, Schemske DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics* **29**: 467–501.

Rich TCG. 2009. Pollen stainability in British *Sorbus* L. (Rosaceae). *Plant Ecology & Diversity* **2**: 85–88.

Rich TCG, Houston L, Robertson A, Proctor MCF. 2010. *Whitebeams, rowans and service trees of Britain and Ireland. A monograph of British and Irish Sorbus L. BSBI: Handbook No. 14*, London: BSBI.

Robertson A, Newton AC, Ennos RA. 2004a. Breeding systems and continuing evolution in the endemic *Sorbus* taxa on Arran. *Heredity* **93**: 487–495.

Robertson A, Newton AC, Ennos RA. 2004b. Multiple hybrid origins, genetic diversity and population genetic structure of two endemic *Sorbus taxa* on the Isle of Arran, Scotland. *Molecular Ecology* **13**: 123–134.

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024

Robertson A, Rich TCG, Allen AM, *et al.* 2010. Hybridization and polyploidy as drivers of continuing evolution and speciation in *Sorbus*. *Molecular Ecology* **19**: 1675–1690.

Sabara HA, Kron P, Husband BC. 2013. Cytotype coexistence leads to triploid hybrid production in a diploid–tetraploid contact zone of *Chamerion angustifolium* (Onagraceae). *American Journal of Botany* **100**: 962–970.

Šarhanová P, Vašut RJ, Dančák M, Bureš P, Trávníček B. 2012. New insights into the variability of reproduction modes in European populations of *Rubus* subgen. *Rubus*: how sexual are polyploid brambles? *Sexual Plant Reproduction* **25**: 319–335.

Scott RJ. 2007. Polyspermy in apomictic *Crataegus*: yes and no. *New Phytologist* **173**: 227–230.

Scott RJ, Spielman M, Bailey J, Dickinson HG. 1998. Parent-of-origin effects on seed development in *Arabidopsis thaliana*. *Development* **125**: 3329–3341.

Siljak-Yakovlev S, Pustahija F, Šolić EM, *et al.* 2010. Towards a genome size and chromosome number database of Balkan flora: C-values in 343 taxa with novel values for 242. *Advanced Science Letters* **3**: 190–213.

Soltis PS, Soltis DE. 2009. The role of hybridization in plant speciation. *Annual Review of Plant Biology* **60**: 561–588.

Suda J, Krahulcová A, Trávnícek P, Krahulec F. 2006. Ploidy level versus DNA ploidy level: an appeal for consistent terminology. *Taxon* **55**: 447–450.

Talent N, Dickinson TA 2007*a*. Endosperm formation in aposporous *Crataegus* (Rosaceae, Spiraeoideae, tribe Pyreae): parallels to Ranunculaceae and Poaceae. *New Phytologist* **173**: 231–249.

Talent N, Dickinson TA. 2007*b*. The potential for ploidy level increases and decreases in *Crataegus* (Rosaceae, Spiraeoideae, tribe Pyreae). *Canadian Journal of Botany* **85**: 570–584.

Uozu S, Ikehashi H, Ohmido N, Ohtsubo H, Ohtsubo E, Fukui K. 1997. Repetitive sequences: cause for variation in genome size and chromosome morphology in the genus *Oryza*. *Plant Molecular Biology* **35**: 791–799.

Vinkenoog R, Bushell C, Spielman M, Adams S, Dickinson HG, Scott RJ. 2003. Genomic imprinting and endosperm development in flowering plants. *Molecular Biotechnology* **25**: 149–184.

Warburg EF, Kárpáti ZE. 1993. *Sorbus* L. In: *Flora Europaea, Volume 2, Rosaceae to Umbelliferae*. TG Tutin, VH Heywood, NA Burges, *et al*., eds. Cambridge: Cambridge University Press, 67–71.

Whitton J, Sears CJ, Baack EJ, Otto SP. 2008. The dynamic nature of apomixis in the angiosperms. *International Journal of Plant Sciences* **169**: 169–182.

Downloaded from https://academic.oup.com/aob/article/116/2/301/180558 by guest on 23 April 2024