Speciation by triparental hybridization in genus *Sorbus* (Rosaceae)

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Abstract

Hybridization associated with polyploidization and apomixis is a frequent mechanism of speciation. *Sorbus* is a genus with ongoing hybridization resulting in a polyploid complex with different parental lineage. *Triparens* is the smallest hybridogenous subgenus of *Sorbus* so far known to comprise only two taxa, *S. intermedia* and *S. × liljeforsii* that combine the genomes of three taxa (*S. aria* agg., *S. aucuparia* and *S. torminalis*). To elucidate the origins of *S. dacica*, *S. paxiana* and *S. tauricola*, three new trigonomic candidates formerly believed to be of biparental origin with either *S. aria* agg. × *S. aucuparia* or *S. aria* agg. × *S. torminalis* lineage we combined data from HPLC and chloroplast DNA analysing additional 33 related taxa as well. We concluded that the ‘*torminalis*-type’ flavonoid profile and the ‘*aucuparia*-type’ plastid indicate the participation of both *S. torminalis* and *S. aucuparia* resulting in the formation of *S. dacica*, *S. paxiana* and *S. tauricola*. *Sorbus aria* agg. as the third ancestor and as a necessary link to meet genes of *S. torminalis* and *S. aucuparia* in one genome is obvious from morphological features (densely tomentose undersides of leaves). The tetraploid cytotypes and obligate pseudogamy of *S. dacica* and *S. paxiana* were determined by flow cytometry and are published here for the first time. The most probable evolutionary scenario for *Triparens* species is: 1. a diploid sexual *S. aucuparia* as pollen acceptor hybridized with a tetraploid apomictic taxon from the *S. aria* agg. producing a triploid apomictic taxon with ‘*aucuparia*-type’ plastid inherited maternally; 2. during a second crossing event this subgenus *Soraria* hybrid as maternal progenitor hybridized with the sexual diploid *S. torminalis* (providing gene(s) of apigenin O-glucuronide synthesis) forming a tetraploid *Triparens* hybrid with ‘*aucuparia*-type’ plastid and ‘*torminalis*-type’ flavonoids.

Keywords Triparental hybridization · Taxonomy · Flavonoids · Chloroplast inheritance · Multiple origin · *Sorbus* subg. *Triparens* · Pseudogamy · Polyploidy

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Introduction

Interspecific hybridization and polyploidization are important drivers of plant evolution and speciation forming new combinations by integrating genetic material mostly of two parent species [56]. The overwhelming majority of the hybridogenous *Sorbus* species are also of biparental origin usually with *S. torminalis* (L.) Crantz or *S. aucuparia* L., more rarely *S. chamaemespilus* (L.) Crantz as maternal parent and a member of *S. aria* agg. as the paternal parent [1, 11, 17, 30, 45, 46, 52, 57, 58, 65] forming the hybridogenous subgenera *Tormaria* Májovský & Bernátová (*S. torminalis* × *S. aria* agg.), *Soraria* Májovský & Bernátová (*S. aucuparia* × *S. aria* agg.) and *Chamaespilaria* Májovský & Bernátová (*S. chamaemespilus* × *S. aria* agg.). There is, however, sporadic evidence for triparental speciation in *Sorbus*. Altogether 11 taxa are known to be triple hybrids, 9 of them are ranked in subgenus *Chamsoraria* Májovský & Bernátová

(*S. chamaemespilus* × *S. aucuparia* × *S. aria* agg.) [3, 44], whereas currently *S. intermedia* (Ehrh.) Pers. and *S. × liljeforsii* T. Rich are classified as representatives of subgenus *Triparens* M. Lepší & T. C. G. Rich (*S. aucuparia* × *S. aria* agg. × *S. torminalis*) [53–55].

Morphological traits, mainly of leaves and fruits, have long been used to classify hybridogenous *Sorbus* taxa (e.g. [18, 25, 44, 54]). In the 1970s and the subsequent decades thin layer chromatography (TLC) was used as a method for taxonomical evaluation of *Sorbus*. Certain flavone glycosides have long been known as reliable and characteristic taxonomic markers suitable for judging the involvement of putative parent species in hybridogenous speciation. Flavone-C-glycoside vitexin occurs solely in the leaves of *S. torminalis*, *S. chamaemespilus* and their hybrids as well as in *S. umbellata* agg., whilst some flavone-O-glycosides (luteolin 7-O-rhamnosylglucoside, luteolin 7-O-diglucoside, luteolin 7-O-glucoside, luteolin 4'-O-glucoside, apigenin 7-glucoside) are exclusive components of *S. torminalis* and its derivatives [7–10, 26].

In the course of studying both herbarium and living material of European *Sorbus*, beside the known *S. intermedia* and *S. × liljeforsii*, its backcross to *S. aucuparia*, three further species, *S. dacica* Borbás, *S. paxiana* Jáv. and *S. tauricola* Zaik. ex Sennikov presumed formerly to be of biparental origin are hypothesized here to have evolved by triparental hybridization. All of them combine morphological features of the three supposed ancestors: (i) the densely tomentose abaxial leaf side of *S. aria* agg., (ii) the greenish-yellowish colour of leaf underside (iii) and the brownish tinge of their fruits during ripening seen in *S. torminalis*, (iv) the more or less separated lower leaflet pair at least in some leaves of the long sterile shoots that indicates genetic involvement of *S. aucuparia*.

Sorbus dacica, a stenoendemic species of Transylvania, Romania, was described from the Trascăului Mountains [4, 5]. It has usually been suggested to have evolved by biparental hybridization between *S. aria* agg. and *S. aucuparia* [4, 21, 24, 25, 61, 63], but Pax [48] suspecting a trigeneric parentage suggested it arose as *S. mougeotii* × *S. torminalis* hybrid (*S. mougeotii* Soy.-Will. & Godr. is well known to have *S. aria* agg. × *S. aucuparia* origin).

Sorbus paxiana is a local endemic of the Cerna Mountains, South-eastern region of Banat, Romania [22]. Opinions are divided about its putative origin. Pax [48] who first published its occurrence considered it to be a hybrid of *S. graeca* (Spach) Schauer (belonging to *S. aria* agg.) and *S. torminalis*. Jávorka [21, 22] assumed its triparental derivation indicating either the sympatric *S. graeca*, *S. austriaca* (Beck) Hedl. and *S. torminalis* or *S. borbasii* and *S. torminalis* as the parent species, of which *S. austriaca* and *S. borbasii* have been undoubtedly resulted from *S. aria* agg. × *S. aucuparia* crossing. Later it was believed to have originated via a biparental cross event either

between *S. aria* agg. and *S. aucuparia* [24] or *S. aria* agg. and *S. torminalis* [25, 61].

Sorbus tauricola is an indigenous species of the Crimean Peninsula recognized and published first by Popov in 1959. It has congruently been supposed to have biparental origin with *S. aria* agg. and *S. torminalis* as ancestors and is known to be a tetraploid taxon [51, 60, 61, 66, 68, 69].

The aims of this study were (1) to investigate evidence for triparental origin of *S. dacica*, *S. paxiana* and *S. tauricola* by means of biochemical and molecular methods and (2) based on flow cytometry to assess their ploidy level and reproduction mode, and (3) delineate putative scenarios of their formation.

In a recent taxonomic paper, the non-hybrid *Sorbus* subgenera were interpreted at generic level and for the five hybridogenous subgenera, new generic names were introduced [61]. An alternative recent taxonomic treatment includes *Sorbus* s.l. in *Pyrus* [12]. Since there is as yet no general taxonomic consensus we follow the widely used traditional delimitation and treat our species under *Sorbus* with the corresponding accepted subgeneric names [41, 55].

Materials and methods

Plant material

To embed our 3 new trigeneric candidates, *S. dacica*, *S. paxiana* and *S. tauricola* in a wider taxonomic context we analysed further 33 related taxa collected at 68 different sites across Europe (58 natural growth sites and 10 gardens or streets of 12 countries) and classified in different European *Sorbus* subgenera as well. Thus altogether 146 individuals of 36 taxa were involved in our study including the four 'basic' parents *S. torminalis*, *S. chamaemespilus*, *S. aucuparia* and *S. aria* s.str., as well as their hybridogenous derivatives. 109 individuals were involved in biochemical analyses, 61 individuals were studied molecularly and 35 individuals were examined using flow-cytometry (Table 1). Locality details and methods applied for the single individuals are listed in the Supplementary Table. Except for some taxa where fewer samples were available, we analysed biochemically three or more individuals per taxon, whilst two individuals per taxon were studied molecularly. To reveal their ploidy level and reproduction mode we analysed 21 individuals of *S. dacica* and 13 individuals of *S. paxiana*. To check its ploidy level and reproduction mode one individual of *S. intermedia* was also analysed by flow cytometry. Ploidy level and reproduction mode of *S. tauricola* were not examined in lack of available fresh leaf and fruit material. Voucher herbarium specimens were deposited in the private herbarium of the first author.

Biochemical studies

To evaluate the possible role of *S. torminalis* in the evolution of *S. dacica*, *S. paxiana* and *S. tauricola* we conducted HPLC-ESI-qTOFMS analysis comparing their apigenin O-glucuronide and vitexin content with the putative parents as well as numerous closely related taxa both morphologically undoubtedly having and apparently lacking *S. torminalis* among the ancestors.

Acetonitrile and methanol (Prolabo HiPerSolv, VWR International, Radnor, PA, USA) were super gradient grade. Formic acid (~98% for mass spectrometry) was obtained from Fluka (Sigma-Aldrich, St. Luis, MO, USA). Crystalline reference substance of vitexin was purchased from Sigma-Aldrich (Budapest, Hungary). A Milli-Q ultrapure water system (Merck Millipore, Billerica, MA, USA) was used throughout the study.

Sample preparation

100 mg sample of pulverized *Sorbus* leaves was weighed into PP tubes, and 5 mL 60% aqueous MeOH containing 1% formic acid and 25 µL of 1000 µg mL⁻¹ daidzein surrogate standard was added to each tube. Samples were extracted for 40 min in an ultrasonic bath (<35 °C at the end). Extracts were centrifuged for 10 min at 8000 g, and 2.5 mL supernatant was transferred to another PP tube and diluted to 10 mL with water. Finally, samples were filtered through a 0.22 µm PTFE membrane before injecting to the HPLC.

Chromatographic separation

Chromatographic separation was carried out on a Phenomenex Kinetex C18, 4.6 × 150 mm, 2.6 µm column (Phenomenex, Macclesfield, UK) using an Agilent 1200 series high performance liquid chromatography (HPLC) system (Waldbronn, Germany). For the chromatography, 0.5% (v/v) formic acid in water (mobile phase A) and 0.5% (v/v) formic acid in acetonitrile (mobile phase B) were used as solvents at a flow rate of 500 µL/min. For the qualification of polyphenolic compounds gradient program started at 8% B, and after 5 min of isocratic run, solvent B was increased linearly and reached 45% at 35 min and then 100% at 40 min. Finally, 100% B was kept constant for 5 min, followed by 15 min isocratic re-equilibration for initial conditions. For the quantification of apigenin derivatives gradient program started at 25% B, and after 1 min of isocratic run, solvent B was increased linearly and reached 44% at 8 min and then 100% at 9 min. Finally, 100% B was kept constant for 2 min, followed by 15 min isocratic re-equilibration for initial conditions.

HPLC-ESI-qTOFMS analysis

The HPLC system including a diode array detector (DAD) was coupled to an Agilent (Santa Clara, CA USA) 6530 quadrupole—time-of-flight mass spectrometer (q-TOFMS), which was equipped with a dual spray ESI source. The q-TOFMS was used with the following operation parameters: capillary voltage, 4000 V; nebulizer pressure, 40 psig; drying gas flow rate, 13 L/min; gas temperature, 350 °C; skimmer voltage, 65 V. During the experiment fragmentor voltage was triggered automatically between 160 and 210 V in positive mode and 140 and 240 V in negative mode. The lower value represents mild conditions in order to minimize in-source fragmentation, while the higher one is to foster in-source fragmentation. Full-scan mass spectra in the range of m/z 50–1100 were recorded at 1.5 spectra/s scanning speed at all times during the chromatographic run. The instrument performed the internal mass calibration automatically, using an automated calibrant delivery system, which introduces the flow from the outlet of the chromatograph together with a low-flow (approximately 10 µL/min) of a calibrating solution. The solution contains the internal reference of HP-921 [hexakis-(1H,1H,3H-tetrafluoro-pentoxo)-phosphazene] and purine. Protonated molecules of purine ([C₅H₅N₄]⁺ at m/z 121.0509) and HP-0921 ([C₁₈H₁₉O₆N₃P₃F₂₄]⁺ at m/z 922.0098) were used as reference masses in positive ion mode, while deprotonated purine at m/z 119.0363 and the formic acid adduct of HP-0921 ([C₁₉H₁₉O₈N₃P₃F₂₄]⁻ at m/z 966.000725) were used for the same purpose in negative ion mode. The DAD was acquiring data in the range of 200–800 nm in 2-nm steps at 0.5 spectra/s acquisition speed. The quantification of vitexin (apigenin-8-C-glucoside) was carried out using reference standard and the standard addition calibration technique, the MS was used in TOF only mode and was set into the extended dynamic range instrument state. In absence of the other apigenin-glycoside (apigenin O-glucuronide) reference standard only the relative peak areas were compared. The relative areas were calculated as compound peak area/daidzein peak area.

Molecular studies

To test whether the 3 triparental candidates *S. dacica*, *S. paxiana* and *S. tauricola* possess ‘*aucuparia*-type’ plastid two cpSSR primer pairs were used comparing the fragment lengths with those of the putative parents as well as several closely related taxa both undoubtedly having and apparently lacking *S. aucuparia* among the ancestors.

DNA extractions were made from silica-dried leaves using a modified CTAB protocol [15, 67]. Amplification of two variable chloroplast DNA regions was carried out by using rpl16 pm1 and trnT-L pm1 primer pairs targeting rpl16 gene intron as well as intergenic spacer between

trnT (UGU) exon and trnL (UAA) 5' exon following Chester et al. [11] (Table 2). The PCR mixture (total volume, 25 µL) contained 1 U Taq DNA Polymerase (Thermo Scientific, Inc.), 2.5 µL 10× reaction buffer (Thermo Scientific, Inc.), 0.2 mM dNTP Mix (Thermo Scientific, Inc.), 2.5 µM each primer and 20–80 ng genomic DNA. The polymerase chain reaction (PCR) was carried out in an Esco Aeris™ Thermal Cycler (Esco Healthcare Pte, Singapore) using the following heating profile: 4 min at 94 °C (initial denaturing step) followed by 32 cycles of 0.5 min at 94 °C (denaturing step), 0.5 min at 50 °C, 1 min at 72 °C (extension step) with an additional 8 min at 72 °C (final extension). PCR products were separated on 1% agarose gels (1 g agarose dissolved in 100 mL 1×TBE solution). Gels were stained with Gel-Red (Biotium, Fremont, CA, USA) and visualized under UV light. PCR fragments checked with gel electrophoresis were analysed by using ABI 3100 (Applied Biosystems, Foster City, CA, USA) capillary gel electrophoresis system adding to ROX 500 an internal lane size standard (Applied Biosystems) to the mix (2 µL PCR product, 0.5 µL internal size standard, 10 µL HiDi formaldehyde). For evaluation of chromatograms Peak Scanner v1.0 (Thermo Fisher Scientific, MA, Waltham, USA) software was applied.

Flow cytometry

Estimation of somatic DNA ploidy level

DNA ploidy level of 35 adult trees (13 individuals of *S. paxiana*, 21 individuals of *S. dacica* and 1 individual of *S. intermedia*) was assessed by flow cytometry with DAPI (4',6-diamidino-2-phenylindole) as a fluorescent stain. *Bellis perennis* L. (2C = 3.38 pg; [59]) was used as an internal standard. Preparation of samples generally followed a two-step procedure described by Doležal et al. [14]. Since only dried herbarium material was available we have not analysed *S. tauricola*.

Approximately 0.5–1 cm long part of a petiole was chopped together with an appropriate volume of fresh leaf tissue of the internal standard in a Petri dish containing 0.5 mL Otto I buffer (0.1 M citric acid monohydrate, 0.5% Tween 20; [47]). The nuclear suspension was then filtered through a 42 µm nylon mesh and the samples were incubated for 10 min at room temperature, a staining solution consisting of 1 mL of Otto II buffer (0.4 M Na₂HPO₄·12 H₂O; [47]) supplemented with DAPI (final concentration 4 µg/mL; Sigma, Steinheim, Germany) and 2-mercaptoethanol (2 µL/mL; Fluka, Buchs, Germany) was added. After 5 min of incubation at room temperature, fluorescence concentration was measured on a CyFlow ML (Partec GmbH, Münster, Germany) equipped with a UV LED 365 nm. Fluorescence concentration of 3000 particles was recorded and fluorescence histograms were further analysed using FloMax

2.0 software (Partec GmbH, Germany). Ploidy levels were deduced based on the previous karyological studies of the genus *Sorbus* [31, 32].

Determination of reproduction modes

The modes of reproduction were determined based on the flow cytometric seed screen [42]. 9 seeds of one mother for *S. paxiana*, 54 seeds of 5 mothers for *S. dacica* and 31 seeds of one mother for *S. intermedia* were analysed. Each seed was analysed separately and only the lateral part of the seed was cut and used for the analysis. The rest of the procedure generally copied the one used for the leaf analysis however some small modifications were made. *Bellis perennis* or *Carex acutiformis* (2C = 0.82 pg; [38]) were used as internal standards; the genome sizes were always recalculated to *B. perennis* using the ratio 3.319 (*B. perennis*/*C. acutiformis*) based on several simultaneous measurements of these two standards in different days. The volume of 0.7 mL of the buffer Otto I was used. After the filtration of the sample, incubation time was prolonged up to 30 min. Then 0.5 mL of the sample's supernatant was refiltered and immediately mixed with the staining solution and measured. Modes of reproduction were inferred based on DNA ploidy levels of embryo and endosperm following Talent and Dickinson [64].

Results

Biochemical studies

Apigenin O-glucuronide was found to be a characteristic flavonoid component occurring exclusively in the leaves of *S. torminalis* and its progenies. The highest concentrations of apigenin O-glucuronide was detected in *S. torminalis*, whilst its putative descendants (taxa grouped in subgenus *Tormaria* and subgenus *Triparens*) also contained it in different and considerably smaller amounts. Of the subgenus *Tormaria* species, *S. gayeriana* Kárp. had the highest concentration. As far as subgenus *Triparens* is concerned we measured the highest concentration in *S. intermedia* and the lowest one in *S. paxiana* (see Table 3 for details).

Of the European 'basal' *Sorbus* species examined, vitexin occurred exclusively in the leaves of *S. torminalis* and *S. chamaemespilus* and their descendants (each taxon of the biparental subgenus *Tormaria* and subgenus *Chamaespilaria* in addition to the trigonometric subgenus *Triparens*). *S. aria* s.str. and *S. aucuparia* both lacked vitexin. The highest concentration was measured in *S. chamaemespilus* and its hybrid, *S. ×ambigua* (Decne.) Beck. Taxa belonging to subgenus *Aria* proved to be rather heterogenous concerning their vitexin content. We did not detect vitexin in the leaves

of *S. danubialis* (Jáv.) Prodan, *S. graeca* agg. and *S. ulmifolia* Kárp., whereas all morphotypes of *S. umbellata* agg. as well as *S. domugledica* Kárp., *S. pannonica* Kárp. and *S. vajdae* Boros contained this compound, in the case of the last three taxa suggesting their possible *S. umbellata* origin.

Species of subgenus *Tormaria* contained vitexin in various amounts. *S. semiincisa* Borbás was found to contain the highest amount, whereas no vitexin was detected in *S. pelsoensis*. As we expected, subgenus *Triparens* taxa also

contained vitexin. *S. tauricola* had vitexin in highest concentration, whereas it was present in *S. paxiana* and *S. intermedia* in smaller amount. In *S. dacica* it was found only in a trace amount (see Table 3 for details).

Molecular studies

Regarding the putative ancestors of subgenus *Triparens* species, fragment length of *S. torminalis* and each taxon

Table 1 Number of individuals of *Sorbus* taxa included in biochemical (HPLC), molecular (cpSSR) and flow cytometry (FCM) analyses

Subgenus/ nothosubgenus	Taxon	Biochemical studies (HPLC)	Molecular studies (cpSSR)	Flow cytometry (FCM)
Torminaria	<i>S. torminalis</i>	3	1	
Chamaemespilus	<i>S. chamaemespilus</i>	3	1	
Sorbus	<i>S. aucuparia</i>	3	1	
Aria	<i>S. aria</i> s.str.	3	1	
	<i>S. danubialis</i>	4	2	
	<i>S. domugledica</i>	2	2	
	<i>S. graeca</i> agg.	3	2	
	<i>S. pannonica</i>	3	2	
	<i>S. ulmifolia</i>	3	2	
	<i>S. umbellata</i> agg.	13	1	
	<i>S. vajdae</i>	3	2	
Chamaespilaria	<i>S. × ambigua</i>	3	2	
Soraria	<i>S. arranensis</i>	1	1	
	<i>S. austriaca</i>	3	2	
	<i>S. borbasii</i>	3	2	
	<i>S. hazslinszkyana</i>	3	2	
	<i>S. hohenesteri</i>	1	1	
	<i>S. mougeotii</i>	2	2	
	<i>S. pseudofennica</i>	1	1	
	<i>S. pseudothuringiaca</i>	1	1	
	<i>S. pulchra</i>	1	1	
	<i>S. × thuringiaca</i>	3	2	
	<i>S. transylvanica</i> n.p.	4	2	
	<i>S. tuzsoniana</i>	3	2	
Tormaria	<i>S. badensis</i>	2	2	
	<i>S. bakonyensis</i>	3	2	
	<i>S. balatonica</i>	2	2	
	<i>S. bohemia</i>	2	2	
	<i>S. gayeriana</i>	3	2	
	<i>S. pelsoensis</i>	3	2	
	<i>S. pseudolatifolia</i>	3	2	
	<i>S. semiincisa</i>	4	2	
Triparens	<i>S. dacica</i>	5	2	21
	<i>S. intermedia</i>	3	1	1
	<i>S. paxiana</i>	6	2	13
	<i>S. tauricola</i>	1	2	
Total:	36	109	61	35

of subgenus *Aria* in *rpl16* locus proved to be quite similar falling into the range of 142–146 bp, whilst *trnT-L* locus showed length of 272 bp. For *S. aucuparia* as the third presumed parent species we found 110 bp and 238 bp in *rpl16* and *trnT-L* loci, respectively.

Each taxon of the hybridogenous subgenus *Soraria* had 109–111 bp in *rpl16* locus, while in *trnT-L* the fragment length was uniformly 238 bp. All species of subgenus *Triparens* were found to have similarly 238 bp in *trnT-L* locus. Except 112 bp in *S. paxiana*, all the remaining three species *S. dacica*, *S. intermedia*, *S. tauricola* were represented with 111 bp in *rpl16* locus. Subgenus *Tormaria* represents 141–146 bp for *rpl16* and 272–273 bp for *trnT-L* loci. *S. chamaespilus* as well as its derivative *S. ×ambigua* were characterised by 141 bp as well as 271–272 bp for *rpl16* and *trnT-L* loci, respectively (Table 3).

Flow cytometry

Estimation of somatic DNA ploidy level

The two potentially trihybrid species analysed for ploidy level, *S. dacica* and *S. paxiana* were tetraploid. Our measurement of one mother plant of *S. intermedia* confirmed it was tetraploid as well (see Table 4 for details).

Determination of reproduction modes

Seed analysis revealed only tetraploid embryos in all three studied taxa (*S. dacica*, *S. paxiana* and *S. intermedia*). The endosperm/embryo ratios indicate pseudogamous reproduction mode of all three species (see Table 5 for details). The pollen contribution to endosperm development was exclusively tetraploid in the case of *S. intermedia* (4x embryo + 12x endosperm), mostly tetraploid (4x + 12x) and rarely diploid (4x + 10x) in *S. dacica* and *S. paxiana*. We therefore conclude that these species are either self-pollinated (by reduced or unreduced pollen) or received unreduced pollen from sympatric diploid species (possibly *S. torminalis*). One seed of *S. intermedia* and two seeds of *S. dacica* contained 16x endosperm, indicating three tetraploid polar nuclei involvement. Triploid pollen contribution which was observed in one *S. paxiana* seed was probably obtained from a co-occurring triploid species.

Discussion

Taxonomic, chorological and nomenclatural notes

Certain flavone-O-glycosides have been known for a long time as exclusive biochemical markers of *S. torminalis* and its hybrids with *S. aria* agg. (subgenus *Tormaria*). Several

taxa belonging to subgenus *Tormaria* have been examined and all of them proved to contain apigenin O-glucuronide confirming the involvement of *S. torminalis* in their origin [8, 10, 26]. Our results are in agreement with these earlier observations and we also detected apigenin O-glucuronide in all subgenus *Tormaria* species studied, providing supporting evidence that *S. torminalis* was one of their parents. In accordance with these previous studies we did not detect apigenin O-glucuronide in any representatives of *Aria*, *Sorbus*, *Chamaespilus*, *Chamaespilaria* and *Soraria* subgenera.

We also found apigenin O-glucuronide in *S. intermedia*, also detected by Challice and Kovanda [8], reflecting its close affinity to *S. torminalis*. *S. tauricola* formerly unambiguously classified in subgenus *Tormaria* [51, 60, 61, 66, 68, 69] was expected to have at least some content of apigenin O-glucuronide. This result supports its putative *S. torminalis* origin.

Despite some clear morphological features suggesting its feasible *S. torminalis* parentage (greenish-tomentose leaf underneath, orangey tinge of fruits), *S. dacica* has been widely considered to belong to subgenus *Soraria* [4, 6, 18, 21, 24, 25, 61, 63]. The presence of apigenin O-glucuronide in the leaves of *S. dacica* supports the hypothesis that *S. torminalis* must be one of its parents. A *S. torminalis* lineage of *S. paxiana* has long been the subject of debate [21, 22, 24, 25]. Nevertheless, the apigenin O-glucuronide content of both species shows clearly the key role of *S. torminalis* as one progenitor in their formation.

Vitexin is a less specific chemotaxonomic marker in *Sorbus* occurring equally in subgenus *Chamaespilaria* (*S. chamaespilus*), subgenus *Torminaria* (*S. torminalis*), subgenus *Aria* (*S. umbellata* agg.) and in their hybrids [8–10, 26]; our results confirmed these published findings. We found that species of subgenus *Tormaria* contained vitexin in various amounts and in case of *S. pelsoensis* we did not detect any. Lack of vitexin in some subgenus *Tormaria* taxa was noticed by Challice and Kovanda [8, 10] and Kézdy [26] as well. This phenomenon can be explained with back-cross processes towards the parent species during which the genes encoding production of flavone-C-glycoside might have been segregated from those encoding synthesis of flavone-O-glycosides. Contrary to the results of Challice and Kovanda [8] and Kézdy [26] we measured vitexin in each subgenus *Tormaria* taxon they also studied (*S. bohémica* Kovanada, *S. bakonyensis* (Jáv.) Kárp., *S. semiincisa*), even though in some cases only a trace amount. It might be interpreted with the higher resolution of HPLC–MS technology as opposed to PC or TLC methods they used. Concerning subgenus *Triparens* we also observed vitexin content in *S. intermedia* which is in concordance with earlier chemotaxonomic survey [8]. Vitexin detected in *S. dacica*, *S. paxiana* and *S. tauricola* also appears to confirm the involvement of *S. torminalis* as parental species in their formation since *S.*

Table 2 Description of the cpSSR primers used in the present study

Primer name	Primer sequence	Plastid DNA region	Repeat type	Size range
rpl16pm1F	GTCATATCATTTGTAGCAACTG	rpl16 intron	(A) ₁₂	110–146
rpl16pm1R	TTCTATCTTTTCTCTCACCCCT			
trnT-Lpm1F	CATTACAAATGCGATGCTCT	trnT-L spacer	(A) ₄ (TA) ₂	241–277
trnT-Lpm1R	CGCTATATTAATAGGTATGTT			

chamaemespilus does not occur sympatrically with either of them and *S. umbellata* agg. is found to co-exist only with *S. paxiana* ([21, 25], Supplementary table).

The molecular results are also in agreement with previous studies [11, 28, 29, 65]. Probably due to the comparatively distant relationship of the compound leaved *S. aucuparia* and the other, simple-leaved ‘basic’ European *Sorbus* taxa *S. torminalis*, *S. chamaemespilus* and *S. aria* agg. its plastid genome appears to be considerably distinct. Hence chloroplast markers are very useful for analysing the involvement of *S. aucuparia* in hybridization processes, of course only in case *S. aucuparia* is the pollen acceptor because of the maternal inheritance of chloroplast genomes. The conspicuously characteristic ‘*aucuparia*-type’ fragment length of the two examined chloroplast loci in all studied subgenus *Soraria* taxa provides evidence for the key role of *S. aucuparia* in the speciation. Nevertheless, since plastid DNA is known to be of maternal inheritance in angiosperms, our cpDNA analyses proved that *S. aucuparia* tends to take part in hybridization processes as pollen acceptor, receiving pollen from a member of the *Aria* group (subgenus *Aria*). This observation is in line with the results of other works which found that *S. aria* agg. is the most common pollen donor in hybridizations resulting in subgenus *Soraria* taxa [11, 17, 45]. The ‘*aucuparia*-type’ plastid of proved (*S. intermedia*) and putative (*S. dacica*, *S. paxiana* and *S. tauricola*) subgenus *Triparens* taxa also indicates their *S. aucuparia* origin and the participation of *S. aucuparia* in the hybridogenous speciation as maternal line.

Our flow cytometric results confirmed the long known tetraploidy and pseudogamy of *S. intermedia* [20, 33, 35–37, 45, 49, 50], on the other hand demonstrated tetraploid cytotype and pseudogamous apomixis in case of *S. dacica* and *S. paxiana*.

The ‘*aucuparia*-type’ chloroplast and the ‘*torminalis*-type’ flavonoid content revealed that *S. dacica*, *S. paxiana* and *S. tauricola* must have genetic material inheriting both from *S. aucuparia* and *S. torminalis* and must have evolved by trigonomic hybridization. One member of *S. aria* agg. as the third parent is obvious from morphological features such as dense hairiness on the underside of the leaves. Furthermore direct hybrids between *S. aucuparia* and *S. torminalis* have never been observed. Consequently *S. dacica*, *S. paxiana* and *S. tauricola* should be placed in the triparental subgenus *Triparens*. With this reclassification the known number of taxa of subgenus *Triparens* increased to 5 (Fig. 1) and the distribution includes along with the Scandinavian-Baltic area (*S. intermedia* and *S. ×liljeforsii*) also the Transylvanian (*S. dacica*) and Banatian (*S. paxiana*) regions in Romania, as well as the Crimean Peninsula (*S. tauricola*).

Based on the recently introduced nomenclatural approach by Sennikov and Kurtto [61] and Sennikov [62], all the three-way hybrid taxa between *S. aria* agg., *S. aucuparia*

Table 3 Results of biochemical and molecular studies showing the characteristic apigenin O-glucuronide content of *Sorbus torminalis* and its hybrids as well as the unique plastid type of *Sorbus aucuparia* and its derivatives

Subgenus/ nothosubgenus	Taxon	Range of vitexin content in dried leaves (mg/g)	Average content of vitexin in dried leaves (mg/g)	Range of apigenin O-glucuro- nide content in dried leaves (mil- lions cps)	Average content of apigenin O-glucuron- ide in dried leaves (mil- lions cps)	Type of flavonoids	rpl16 locus (bp)	trnT-L locus (bp)	Type of chlo- roplast
<i>Torminaria</i>	<i>S. torminalis</i>	0.265–0.512	0.368	17.667– 22.634	19.982	TORMI- NALIS	146	272	
<i>Chamae- mespilus</i>	<i>S. chamae- mespilus</i>	2.675–5.935	3.841	nd	nd		141	271	
<i>Sorbus</i>	<i>S. aucuparia</i>	nd	nd	nd	nd		110	238	AUCUPARIA
<i>Aria</i>	<i>S. aria s.str.</i>	nd	nd	nd	nd		142	272	
	<i>S. danubialis</i>	nd	nd	nd	nd		142	272	
	<i>S. domugl- edica</i>	0.054–0.083	0.068	nd	nd		142	272	
	<i>S. graeca agg.</i>	nd	nd	nd	nd		143	272	
	<i>S. pannonica</i>	0.076–0.318	0.189	nd	nd		143	272	
	<i>S. ulmifolia</i>	nd	nd	nd	nd		142	272	
	<i>S. umbellata agg.</i>	0.260–1.015	0.579	nd	nd		142	272	
	<i>S. vajdae</i>	0.029–0.031	0.030	nd	nd		142	272	
<i>Chamaespi- laria</i>	<i>S. × ambigua</i>	0.654–2.583	1.667	nd	nd		141	272	
<i>Soraria</i>	<i>S. arranensis</i>	nd	nd	nd	nd		109	238	AUCUPARIA
	<i>S. austriaca</i>	nd	nd	nd	nd		109	238	AUCUPARIA
	<i>S. borbasii</i>	nd	nd	nd	nd		111	238	AUCUPARIA
	<i>S. hazslinsz- kyana</i>	nd	nd	nd	nd		109	238	AUCUPARIA
	<i>S. hohenes- teri</i>	nd	nd	nd	nd		108	238	AUCUPARIA
	<i>S. mougeotii</i>	nd	nd	nd	nd		109	238	AUCUPARIA
	<i>S. pseu- dofennica</i>	nd	nd	nd	nd		109	238	AUCUPARIA
	<i>S. pseudo- thuringi- aca</i>	nd	nd	nd	nd		109	238	AUCUPARIA
	<i>S. pulchra</i>	nd	nd	nd	nd		110	238	AUCUPARIA
	<i>S. × thuringi- aca</i>	nd	nd	nd	nd		109	238	AUCUPARIA
	<i>S. transyl- vanica n.p.</i>	nd	nd	nd	nd		109	238	AUCUPARIA
	<i>S. tuzso- niana</i>	nd	nd	nd	nd		109	238	AUCUPARIA
<i>Tormaria</i>	<i>S. badensis</i>	0.065–0.134	0.099	1.677–2.377	2.027	TORMI- NALIS	143	273	
	<i>S. bakony- ensis</i>	0.025–0.065	0.047	5.182–7.119	5.951	TORMI- NALIS	144	273	
	<i>S. balatonica</i>	0.076–0.110	0.093	3.035–3.299	3.167	TORMI- NALIS	144	273	
	<i>S. bohémica</i>	0.095–0.143	0.119	0.792–3.151	1.972	TORMI- NALIS	144	273	
	<i>S. gayeriana</i>	0.052–0.435	0.199	8.131–9.553	8.801	TORMI- NALIS	144	273	

Table 3 (continued)

Subgenus/ nothosubgenus	Taxon	Range of vitexin content in dried leaves (mg/g)	Average content of vitexin in dried leaves (mg/g)	Range of apigenin O-glucuronide content in dried leaves (mil- lions cps)	Average content of apigenin O-glucuronide in dried leaves (mil- lions cps)	Type of flavonoids	rpl16 locus (bp)	trnT-L locus (bp)	Type of chlo- roplast
	<i>S. pelsoensis</i>	nd	nd	1.820–7.658	4.523	TORMI- NALIS	143	273	
	<i>S. pseudolatifolia</i>	0.029–0.084	0.057	4.628–6.908	5.427	TORMI- NALIS	146	272	
	<i>S. semiincisa</i>	0.178–0.287	0.235	3.845–6.841	5.346	TORMI- NALIS	141	272	
<i>Triparens</i>	<i>S. dacica</i>	0.010–0.055	0.029	1.832–3.398	2.530	TORMI- NALIS	111	238	AUCUPARIA
	<i>S. intermedia</i>	0.092–0.166	0.130	3.439–5.337	5.315	TORMI- NALIS	111	238	AUCUPARIA
	<i>S. paxiana</i>	0.031–0.484	0.206	0.102–2.430	0.787	TORMI- NALIS	112	238	AUCUPARIA
	<i>S. tauricola</i>	0.551	0.551	3.718	3.718	TORMI- NALIS	111	238	AUCUPARIA

nd not detected

Table 4 Results of flow cytometric analyses of *S. dacica*, *S. paxiana* and *S. intermedia* leaves; *Bellis perennis* was used as the internal standard

Taxon	<i>S. intermedia</i>	<i>S. dacica</i>	<i>S. paxiana</i>
Number of individuals analysed	1	21	13
Observed sample/standard ratio	0.78	0.75–0.79	0.77–0.79
Average coefficient of variation of the sample	2.81	2.64	2.79
Average coefficient of variation of the standard	1.77	2.87	2.35
Estimated DNA ploidy level	2n ~ 4x	2n ~ 4x	2n ~ 4x

Table 5 Flow cytometric seed screening results for *S. dacica*, *S. paxiana* and *S. intermedia* seeds; *Bellis perennis* (B) or *Carex acutiformis* (X) were used as internal standards

Mother species	Maternal ploidy	Standard	Number of seeds	Embryo/standard ratio	Endosperm/ standard ratio	Estimated ploidy level of the embryo	Estimated ploidy level of the endosperm
<i>S. dacica</i>	4	B	10	0.77–0.80	1.94–2.06	4	10
		X	1	2.53	6.32	4	10
		B	15	0.77–0.79	2.28–2.45	4	12
		X	26	2.50–2.72	7.38–8.18	4	12
		B	2	0.76–0.77	3.02–3.04	4	16
Total			54				
<i>S. paxiana</i>	4	B	2	0.79	1.99–2.02	4	10
		B	1	0.8	2.19	4	11
		B	4	0.79–0.81	2.34–2.42	4	12
		X	2	2.71–2.73	8.01–8.05	4	12
Total			9				
<i>S. intermedia</i>	4	X	30	2.53–2.65	7.71–8.07	4	12
		X	1	2.63	10.58	4	16
Total			31

Fig. 1 Typical leaves of *Sorbus* subg. *Triparens* taxa. **a** *Sorbus intermedia* (from cultivation, Hungary), **b** *Sorbus* × *liljeforsii* (Garwnant Forest Centre, Wales), **c** *Sorbus dacica* (Torda Gorge, Romania), **d** *Sorbus paxiana* (Cerna Valley, Romania), **e** *Sorbus tauricola* (Crimean Mts, Crimean Peninsula) (scale bar: 1 cm)

and *S. torminalis*, namely *S. dacica*, *S. paxiana*, *S. tauricola*, *S. intermedia* and *S. × liljeforsii* would be accommodated in the genus *Scandosorbus* Sennikov (*Scandosorbus* replacing *Borkhausenia* Sennikov & Kurtto for nomenclatural reasons; [62]). This nomenclatural approach is still under debate (see e.g. [34]), but if becomes accepted new combinations will be required for *S. dacica*, *S. paxiana* and *S. tauricola*.

Putative scenarios of *Triparens* speciation

Subgenus *Triparens* comprises apomictic *Sorbus* taxa that combine the genomes of *S. aria* agg., *S. aucuparia* and *S. torminalis*. They have evolved at least by two independent hybridization events. Since the chloroplast genome is maternally inherited in angiosperms including *Sorbus*, and each subgenus *Triparens* species shows ‘*aucuparia*-type’ plastid, *S. aucuparia* must be the ovule donor in at least one hybridization event.

The most probable evolutionary pathway is that as a first step, a diploid sexual *S. aucuparia* as pollen acceptor (contributing with a reduced haploid egg) hybridized with a tetraploid apomictic taxon from the *S. aria* agg. (contributing with a reduced diploid pollen) producing a triploid apomictic subgenus *Soraria* taxon with ‘*aucuparia*-type’ plastid inherited maternally. Subsequently during a second crossing event this subgenus *Soraria* hybrid as maternal progenitor (providing an unreduced triploid egg and ‘*aucuparia*-type’ plastid) hybridized with the sexual diploid *S. torminalis* (providing a reduced haploid pollen containing gene(s) of apigenin O-glucuronide and vitexin

synthesis) leading to the formation of a tetraploid subgenus *Triparens* hybrid with ‘*aucuparia*-type’ plastid and apigenin O-glucuronide content (Fig. 2). Unreduced diploid pollen coming from diploid *S. aria* s.str. is unlikely, which is supported also by the results of Lepší et al. [33] who did not observe unreduced pollen in any studied individuals of diploid sexual *S. aria* s.str. at all. Further support for this scenario is that the diploid *S. aria* s.str. as a potential pollen donor is not native in the Baltic-Scandinavian region where *S. intermedia* has evolved [61]. The tetraploid *S. rupicola* (Syme) Hedl., the only subgenus *Aria* species occurring sympatrically is supposed to be as one of its parents [54]. *S. aria* s.str. has not been reported in the Crimean Peninsula either [61] and only tetraploid members of subgenus *Aria* have been found to co-exist with *S. tauricola* [61, 68, 69]. We have not detected any diploid *S. aria* s.str. growing together with *S. dacica* and *S. paxiana* either, but several tri- and tetraploid representatives of subgenus *Aria* were revealed by flow cytometric cytotype mapping (Nosková et al. unpublished data). This concept is supported also by the fact that at least one subgenus *Soraria* taxon always occur sympatrically with all known subgenus *Triparens* species ([21, 22, 25, 36, 68], Németh et al. unpublished data).

A second possible speciation pathway is that in the first hybridization event a reduced diploid male gamete of a tetraploid *S. aria* agg. fused with a haploid female gamete originating from the diploid *S. torminalis*. In biparental subgenus *Tormaria* *Sorbus aria* s.l. appears to be the most frequent pollen donor in hybridizations [1, 11, 17, 30, 45, 46, 52, 57, 58, 65], contrary to the very rare reverse direction of pollen flow [45, 46]. As a second step the triploid subgenus *Tormaria* progeny as pollen donor with an unreduced triploid pollen fertilizes a haploid reduced female egg of *S. aucuparia* inheriting the chloroplast genes from the latter one and creating a tetraploid trigeneric subgenus *Triparens* species. Lepší et al. [33], however, did not find unreduced pollen among *Sorbus* s.l. they studied at all and even triploids produced reduced pollen. Hence this evolutionary route is much less likely. Moreover, there is no information on any subgenus *Tormaria* taxa as potential medial ancestor co-occurring with any subgenus *Triparens* species ([61], Németh pers. obs.), which also makes this scenario much less probable.

A third theoretic evolutionary pathway is that a member of *S. aria* agg. crossed with an interspecific hybrid of *S. aucuparia* and *S. torminalis*. However, this scenario is very unlikely because the hybridization between *S. aucuparia* and *S. torminalis* is hampered by their total incompatibility in both directions as reported earlier by Baksay [2] and in addition, no hybrid of these two species is known.

A final speculative evolutionary pathway is when a diploid amphimictic subgenus *Soraria* hybrid which produces

Fig. 2 The most probable hypothetical scenario of *Sorbus* subg. *Triparens* speciation via triploid subg. *Soraria* bridge, showing maternal inheritance of ‘*aucuparia*-type’ chloroplast from diploid sexual *Sorbus aucuparia* as well as inheritance of apigenin O-glucuronide and vitexin content from diploid sexual *Sorbus torminalis* in paternal line-

age. The supposed genome composition proposed by Liljefors [37] is indicated with capital letters (A: subgenus *Aria* genome, B: *S. aucuparia* genome, T: *S. torminalis* genome). Additional abbreviations: Sx: sexual, Ap: apomictic

unreduced gamete crosses with an unreduced diploid gamete of an amphimictic subgenus *Tormaria* hybrid. Although this way of gamete fusion is not impossible but very improbable and hybrids with this kind of origin have hitherto not been reported.

Conclusion for future biology

Hybridization accompanied by polyploidisation is regarded as an important process in plant speciation. Allopolyploidy is considered to be a quite frequent phenomenon and plays a very important role in plant evolution usually with the involvement of two related species as parents. However, there is reasonably sporadic evidence for speciation by triparental hybridization in the plant world as a whole. For example, natural trigenomic taxa are known in *Epilobium* (*Epilobium ciliatum* × *E. hirsutum* × *E. parviflorum*, [27]), *Potamogeton* (*Potamogeton perfoliatus* × *P. gramineus* × *P. lucens*, [23]) and *Cardamine* (*Cardamine rivularis* × *C. amara* × *C. pratensis*,

[71]). Artificially produced triparental hybrids were published from the genera *Melilotus* [39], *Rosa* [19], *Triticum* [70], *Allium* [16] and *Brassica* [40]. They almost all belong to herbaceous plants. Although some published data are known about trihybrids in *Salix* and *Populus* (e.g. [13, 43]), except for *Sorbus* s.l. there is no evidence for recent natural trigenomic hybridization and speciation among arboreal plants.

In Europe *Sorbus* is considered to be a very complicated group including plenty of hybridogenous polyploid apomictic species evolved by hybridization of the four obligately or facultatively sexual taxa *Sorbus aria* s.l., *Sorbus chamaemespilus*, *Sorbus torminalis* and *Sorbus aucuparia*. In most cases classification of these nothospecies based on morphological features appears to be quite reliable, occasionally the morphological approach alone can lead to misinterpretation concerning the exact taxonomic position. In this study biochemical and molecular methods are combined to reveal supposed triparental (*S. aria* agg., *S. aucuparia*, *S. torminalis*) origin of three stenoendemic, pseudogamous tetraploid *Sorbus* species formerly believed

to be of biparental lineage (*S. aria* agg., *S. aucuparia* or *S. aria* agg., *S. torminalis*). The presence of both apigenin O-glucuronide and vitexin as flavonoid markers in the leaves of *Sorbus dacica*, *Sorbus paxiana* and *Sorbus tauricola* suggests the involvement of *Sorbus torminalis* in their origin, whilst the characteristic type of their chloroplast clearly shows that *Sorbus aucuparia* also must have been among the parent species.

Our new results confirm the presence of triparental speciation mechanism within a genus which includes a number of taxa with restricted area and high degree of threat. Since hybridization is not only a mechanism of speciation but also a way of environmental adaptation, the recognition of triparental taxa and their studies in depth highly facilitate the understanding of plant responses to environmental changes.

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Authors' contributions CN conceived of the presented idea, collected the vast majority of samples for all analyses, conducted molecular analyses, participated in biochemical analyses, wrote considerable part of the draft of the manuscript, prepared the majority of illustrations and took comments from the co-authors. NP was responsible for biochemical analyses, wrote the biochemical part of 'Materials and methods' section and contributed to the interpretation of the results. JN carried out flow-cytometric analyses, wrote the flow-cytometric part of 'Materials and methods' section, prepared flow-cytometric illustrations and contributed to the interpretation of the results. MH supervised the project.

Data accessibility The datasets supporting this article have been uploaded as part of the Supplementary Material.

Compliance with ethical standards

Conflict of interest We have no competing interests.

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