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Reticulate phylogeny in the genus *Sorbus*: the case of *Sorbus haljamovae* Bernátová et Májovský

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Abstract

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Phylogenetic relationships between diploid sexual species *S. aria* and *S. chamaemespilus* and their putative alloploid hybrid *S. haljamovae* were studied on 8 localities in Central Slovakia. Six polymorphic chloroplast microsatellite markers were used to infer polymorphism levels in parental taxa and direction of hybridization. Six haplotypes were identified in the analyzed material. Haplotype 2 was found exclusively in *S. chamaemespilus*, which was found to be completely monomorphic. Haplotypes 1 and 4 were found only in *S. aria*, haplotypes 3 and 5 were shared by *S. aria* and *S. haljamovae*. A single specimen of *S. aucuparia*, used as outgroup in the phylogenetic analysis, contained a specific, highly divergent haplotype. Based on these results, we suggest that *S. haljamovae* is a polyphyletic taxon originating from at least two independent hybridization events.

Keywords

chloroplast microsatellites, hybridization, reticulate phylogeny, *Sorbus haljamovae*

Introduction

Phylogeny has for long been considered a simple branching process. In most phylogenetic models, a taxon (or, more generally, an operational taxonomical unit) can be linked only to its ancestor (usually a hypothetical taxonomical unit), but the connection between already existing taxa is not allowed. Reticulate relationships among taxa have, however, been recorded in many situations. In the plant kingdom, the most important underlying mechanism is hybridization. Almost 25% of plant species are supposed to be involved in hybridization (Mallet, 2005). Hybridization and introgression are thus rule rather than exception in the plant kingdom and the occurrence of hybrids is by far not limited to specific sites (Arnold, 2006; Cronn & Wendel; 2004; Rieseberg & Carney, 1998). The view of hybridization as a lapsus of nature is shifting towards a full recognition of its role as a creative force in plant evolution and speciation (cf. Wissemann, 2007).

In the case of alloploidy, hybridization is associated with genome duplication. Polyploidy is a common mechanism of the diversification and speciation in plants (Rieseberg, 1997). Most plant taxa have overcome a change of the genome size in their evolutionary past (Soltis et al., 2004). Many but not all alloploid taxa are apomictic, so that complicated hybrid complexes have been formed in several genera (cf. Nelson-Jones et al., 2002).

Reticulate evolution is not limited to the interspecific level. Intraspecific evolution also includes exchange of genetic material among genetically different individuals and populations (Smouse, 2000), which may represent recognized taxonomical units (subspecies, varieties) or populations, whose morphological distinctness need not justify a separate taxonomic classification, but which may be strongly differentiated at adaptive as well as neutral genes.

Alloploidy as a mechanism of speciation has widely occurred in the genus *Sorbus*, where plenty of taxa (usually stendoendemics) have been described, originating putatively from alloploid hybridization (Nelson-Jones et al., 2002). Some taxa were even suggested to be hybrids of three or more species (Bernátová and Májovský, 2003). For our study, we selected one of the pink-flowered hybridogenous taxa, namely *Sorbus haljamovae* Bernátová et Májovský, which, based on morphological assessment, is supposed to have originated from alloploid hybridization between *S. aria* and *S. chamaemespilus* (Bernátová and Májovský, 2003). It occurs mostly in subalpine or dealpine communities with the dominance of *Pinus mugo* on calcareous rocks, the occurrence is limited to high mountain ranges of Central Slovakia (Veľká Fatra, Malá Fatra, Nízke Tatry).

Using maternally inherited markers, we tried to identify which species in the *S. aria – S. chamaemespilus* complex are the maternal ones in the interspecific hybridization, and how the monophyletic or polyphyletic origin of the hybridogenous taxon *S. haljamovae* is reflected in its geographic distribution.

Material and methods

Samples of subalpine *Sorbus* taxa were collected on the summits of mountain ranges of Central Slovakia (Table 1) during 2009–2010. Sampling was done preferably during the flowering seasons to allow reliable determination of the collected specimens, which was done by Dr. D. Bernátová (Botanical Garden of the Comenius University, Blatnica) either directly in the field or subsequently on herbarium specimens. Sampling was completed by herbarium specimens deposited in the Botanical Garden in Blatnica. A single *S. aucuparia* specimen was also included to provide outgroup for the analysis of phylogenetic relationships among chloroplast haplotypes.

Total genomic DNA was isolated from air-dried leaves using CTAB method according to Doyle and Doyle (1987), which was modified for a smaller amount of plant material. For genotyping, six microsatellite loci (*trnT-Lpm4, trnT-Lpm3, rps16pm2, rps16pm1, rpl16pm1, trnT-Lpm1*) according to Chester et al. (2007) were used. Different size of alleles and suitable fluorescent labelling enabled multiplexing primers into one group. All PCRs were performed in 6 µl reactions in a GeneAmp® PCR System 9700 (Applied Biosystems) or iQ5 real-time PCR detection system (Bio-Rad) thermal cycler. PCR Master mix contained approximately 50 ng of DNA, 3 µl Qiagen Multiplex PCR Kit and the following concentrations of primers: 0.4 µM trnT-Lpm4, 0.4 µM trnT-Lpm3, 0.05 µM rps16pm2, 0.05 µM rps16pm1, 0.05 µM rpl16pm1, 0.4 µM trnT-Lpm1. Amplification profile consisted of an initial denaturation step at 95 °C for 15 min, followed by 32 cycles with the following profile: 30 s denaturation at 94 °C, 90 s annealing step at 50 °C and 1 min extension step at 72 °C. Final extension was 8 min at 72 °C. Amplification products were separated on a ABI 3100 DNA sequencer, and genotypes were determined using the GeneMapper software v 3.7.

Distances between haplotypes were assessed as the sums of insertions/deletions over the analyzed microsatellite loci. Although the stepwise mutation model is generally considered more appropriate for tandem-repeat loci than the infinite allele model, size differences of alleles did not indicate stepwise insertions/deletions except in locus 3, with 1 bp size differences. Therefore, we considered that the size difference is equal to the number of mutational events at locus 3, whereas a single indel was supposed to produce alleles at the remaining loci. Phylogenetic relationships among haplotypes were reconstructed using the nearest neighbour clustering.

Haplotypic diversity of taxa at individual locations was assessed by gene diversity (Nei, 1978):
$h_e = 1 - \Sigma_i p_i^2$; p_i being the frequency of the *i*th haplotype.

Geographical distribution of taxa was visualized in the ArcView 3.2 environment (ESRI, Redlands, USA).

Results and discussion

As expected, the number of alleles found at the chloroplast loci under study was generally low (Table 2). In spite of analyzing three generally recognized species

Table 1. Basic characteristics of the sampling locations

Location	Longitude	Latitude	Altitude	Forest type class[1]	Soil type (WRB)[2]
Pekarová	18.9644	48.9572	880	Fagetum delapinum	Litho-Rendzic Leptosol
Poludňové skaly	19.0839	49.2378	1,090	Fagetum delapinum	Rendzic Leptosol
Salatín	19.3628	48.9792	1,600	Mughetum calcicolum	Rendzic Leptosol
Siná	19.5719	49.0002	1,150	Fageto-Piceetum	Rendzic Leptosol
Skalná alpa	19.1919	48.9839	1,440	Mughetum calcicolum	Foli-Rendzic Leptosol
Stratenec	18.9675	49.1794	1,460	Mughetum calcicolum	Litho-Rendzic Leptosol
Čierny kameň	19.1464	48.9378	1,380	Mughetum calcicolum	Rendzic Leptosol
Haľamova kopa	18.9944	48.8986	1,070	Fageto-Piceetum	Rendzic Leptosol

[1]Hančinský (1972)
[2]Fao/Iiasa/Isric/Isscas/Jrc (2009)

belonging to different subgenera, only two to four alleles per locus were found, whereby one allele at each locus was unique for *S. aucuparia*. At the loci *trnT-Lpm4*, *rps16pm2*, *rps16pm1* and *rpl16pm1*, differences among alleles by multiples of the repeat motif length could be identified. However, larger insertions/deletions were present at the loci *trnT-Lpm3*, *rpl16pm1* and *trnT-Lpm1*. As we did not sequence the alleles, we were unable to identify the number of mutation events that could have produced variation at such loci. Therefore, for the sake of the phylogenetic analysis, we considered such alleles separated by a single insertion or deletion event.

Allele combinations resulted in six haplotypes. One, highly divergent, was found only in the *S. aucuparia* specimen, used as outgroup in the phylogenetic analysis. The other putatively diploid sexual species did not share any haplotype. All specimens of *S. chamaemespilus* (represented by the by far biggest sample size

Table 2. Haplotypes and the corresponding allele sizes at chloroplast microsatellite loci found in the studied *Sorbus* taxa

Haplotype	*trnT-Lpm4*	*trnT-Lpm3*	*rps16 pm2*	*rps1 6pm1*	*rpl16 pm1*	*trnT-Lpm1*	Taxon
1	115	164	270	100	143	272	*S. aria*
2	115	164	271	100	143	272	*S. chamaemespilus*
3	115	187	269	100	144	272	*S. aria, S. haljamovae*
4	115	187	270	100	144	272	*S. aria*
5	115	187	271	100	144	272	*S. aria, S. haljamovae*
x	121	162	268	104	112	239	*S. aucuparia*

Table 3. Sample size (*n*) and haplotypic diversity (h_e; Nei 1978) of *Sorbus* taxa at individual locations

Population	*S. chamaemespilus*		*S. haljamovae*		*S. aria*	
	h_e	*n*	h_e	*n*	h_e	*n*
Pekarová	–	–	–	–	0.653	7
Poludňové skaly	–	1	0.000	6	0.000	5
Salatín	0.000	19	–	–	–	1
Siná	0.000	8	0.000	2	0.555	15
Skalná alpa	0.000	21	0.000	10	0.500	2
Stratenec	0.000	2	–	–	0.500	6
Čierny kameň	0.000	16	–	–	–	–
Haľamova kopa	–	–	–	–	0.000	4
Total		67		18		40

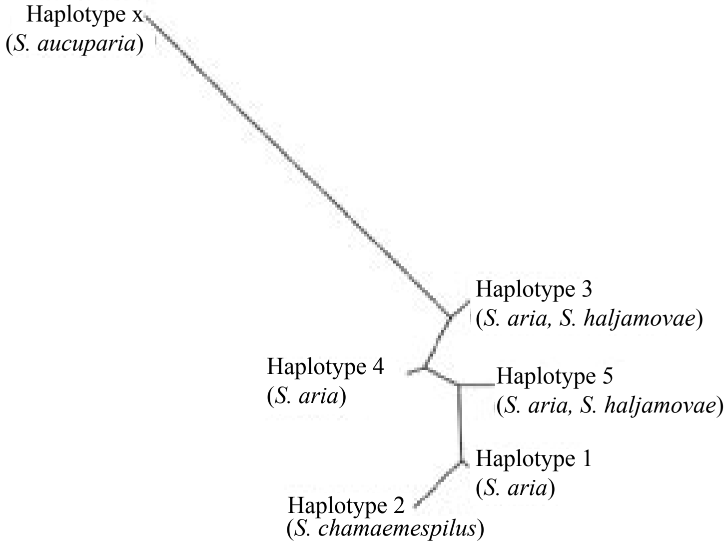

Fig. 1. Phylogenetic relationships (radial tree based on neighbour joining) among chloroplast haplotypes in the *Sorbus* complex.

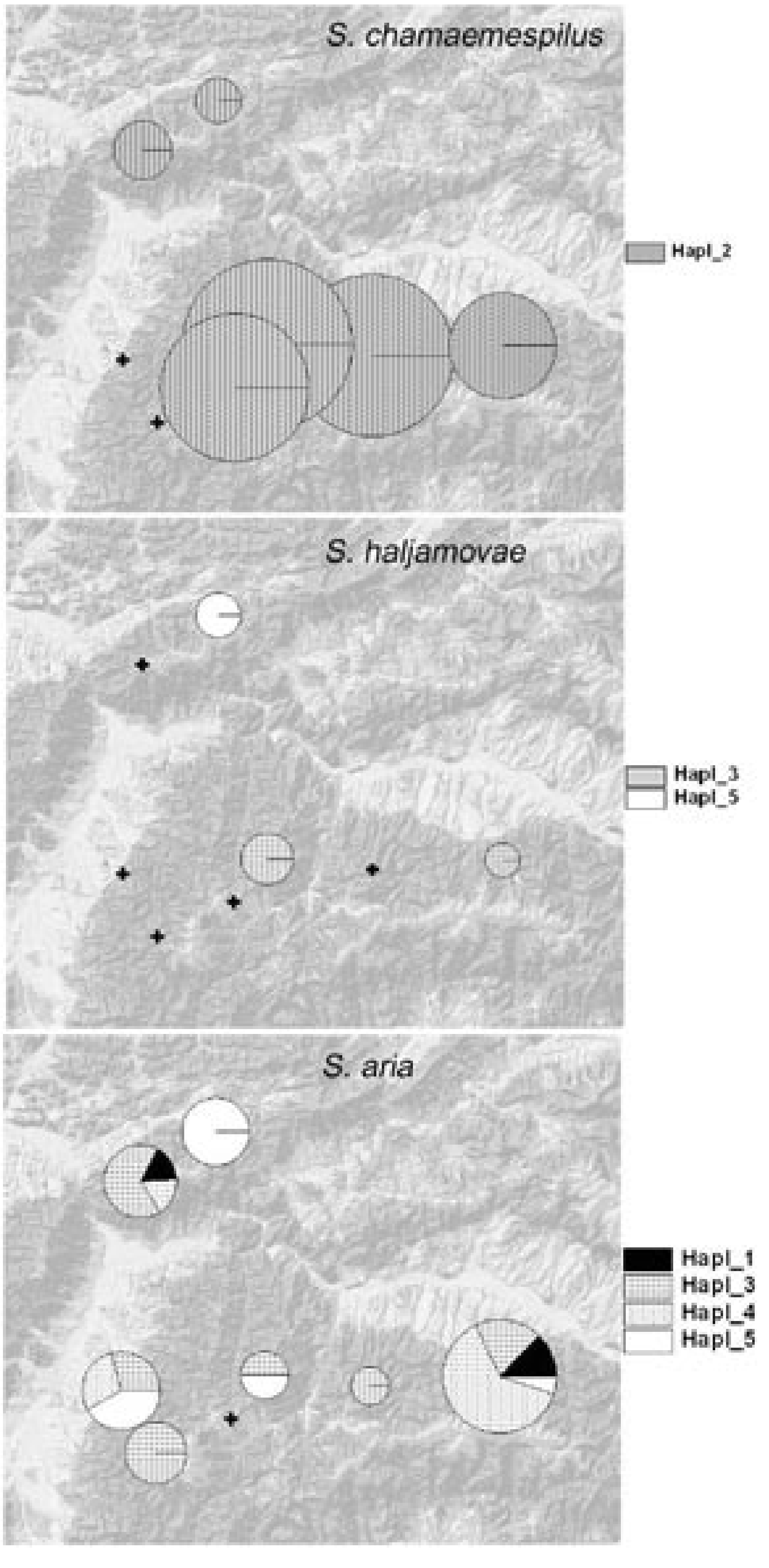

Fig. 2. Distribution of haplotypes in individual populations of *Sorbus* taxa. Symbol size corresponds to sample size, cross means absence of a taxon at the respective location.

in our collection; see Table 3) contained exclusively haplotype 2, which was not found in any other taxon. On the other hand, *S. aria* was found polymorphic, containing 4 haplotypes. Among them, haplotypes 3 and 5 were shared with *S. haljamovae* (Table 2). Phylogenetic relationships among haplotypes, as revealed by the neighbour-joining tree, do not exhibit any clear pattern related to taxonomy (Fig. 1) or geography (Fig. 2).

Central position of *S. aria* (although polymorphic) between *S. aucuparia* and *S. chamaemespilus* (Fig. 1) might reflect its ancient status, also confirmed by the fact, that *S. aria* aggregate is an obligatory participant in all known interspecific crosses in *Sorbus* (Nelson-Jones et al., 2002).

Relatively small sample sizes at many locations do not allow make any definite conclusions about the local polymorphism levels. However, most local populations were monomorphic (Fig. 2). Actually, only *S. aria* exhibited polymorphism at the local population level, with up to four haplotypes per population (Table 3).

Hypothetically, the observed patterns allow several explanations in terms of phylogeny and biogeography, nevertheless, not equally probable:

- Incomplete lineage sorting. Because we did not estimate genome size by flow cytometry, we actually cannot be sure that *S. haljamovae* really is an alloploid hybrid. It may eventually represent another diploid (sexual or apomictic) taxon sharing ancestral polymorphisms with *S. aria*.
- Homoplasy of and/or indpendent mutation events. Because microsatellite alleles represent tandem repeats of short sequence motifs and arise easily through slipping of DNA polymerase during the DNA replication, SSR loci are highly mutable and thus the probability that identical allele sizes are formed by independent insertions or deletions of sequence motifs is not negligible. Moreover, homoplasious alleles may arise by reverse mutations.
- Polyphyletic origin of *S. haljamovae*, i.e. polytopic *in situ* formation of hybrids through independent (alloploid) hybridization events. The fact that *S. haljamovae* shares maternal haplotypes with the local *S. aria* population at each location indicates that *S. aria* contributed by female gametes and *S. chamamespilus* was the father in such natural crosses.

We consider the first hypothesis not very probable. There is a general consent that there are only 5 sexual diploids in the genus *Sorbus* in Europe (Nelson-Jones et al., 2002; Robertson et al., 2010). All pink-flowered taxa or taxonomically unclassified individuals are generally supposed to have originated from hybridization of *S. chamaemespilus* with another taxon (usually *S. aria* has been suggested to contribute by a part of the hybrid genome). Morphologically, at least concerning flower, fruit and leaf traits, *S. haljamovae* represents transition between *S. chamaemespilus* and *S. aria* (Bernátová and Májovský, 2003).

The second concern is more serious. Any phylogenetic analysis is based on the assumption that similarities of traits under study is due to homology, not homoplasy. Admitting that identical allele sizes observed in different taxa result from independent mutations would ruin the relevance of this marker type for the reconstruction of phylogeny in general. However, organellar tandem repeats are commonly used for this purpose (cf. Pleines et al., 2009; Weising and Gardner 1999). In particular, cpSSRs have also successfully been used for the study of alloploid and homoploid hybridization (Angioi et al., 2010; Li et al., 2009; Zeinalabedini et al., 2010), including the genus *Sorbus* (Chester et al., 2007). We do not feel competent to judge the mutation rates of different parts of the chloroplast genome: chloroplast DNA is generally regarded as conservative, but this need not apply to the whole chloroplast sequence and microsatellites may represent mutation hotspots. Nevertheless, although the haplotype divergence within the subgenera *Aria* and *Chamaemespilus* is small, *S. aucuparia* specimen used in our study is characterized by a highly divergent haplotype and the remaining material we analyzed indicates that there is a plenty of haplotypes in the subgenus *Sorbus*, all of them equally genetically distant from those found in *S. aria* and *S. chamaemespilus* (unpublished data). If allele-size sharing by different *Sorbus* taxa is due to random processes of forming new mutations and reverting them to the original state across taxa, then such process should be in action in the whole genus, not only particular subgenera.

Conclusions

We consider polyphyletic origin of *S. haljamovae* the most plausible explanation of the observed haplotype distribution patterns. Why different crossing events produced at least two morphologically homogeneous groups (*S. haljamovae* and *S. zuzanae*) remains a question. We do not have reliable information about reproduction biology of subalpine *Sorbus* taxa, extent of apomixis (suggested by Jankun, 1993, as an important mechanism of evolution in *Sorbus*), ploidy level distribution in the populations of the putative parental and hybridogenous taxa, or back-crossability of hybrids with parental species and thus potential for introgression. No signs of backcrosses have been reported from Slovakia, but this is not a proof of the absence of introgression.

There remains thus much unknown about the formation of putatively hybridogenous taxa in subalpine *Sorbus*. In addition to fundamental scientific importance for biology, the information about reticulate phylogeny patterns is relevant for practice in the case of trees. For acquiring of forest reproductive material and designing appropriate measures of gene conservation (mainly *in situ*), the extent and direction of introducing alien genes in the gene pool of a population should be known. Another aspect of relevance for society is nature conservation. Frequently, a single alloploid apomictically propagating genotype was classified as a species. Because of rarity, such taxa frequently enjoy protection at the species level, which is not reasonable in this case. Elucidation of mechanisms of hybrid speciation and preservation of species integrity may thus be relevant for the elaboration of effective approaches to species conservation.

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Retikulátna fylogenéza v rode *Sorbus*: prípad *Sorbus haljamovae* Bernátová et Májovský

Súhrn

Sledovali sme fylogenetické vzťahy v rámci hybridného komplexu *S. aria – S. chamaemespilus*. Taxón *S. haljamovae* je považovaný za aloploidného hybrida oboch diploidných a pohlavne sa množiacich rodičovských druhov. Materiál (celkovo 135 jedincov) bol zozbieraný resp. získaný z 8 lokalít vysokých pohorí stredného Slovenska. Na identifikáciu hybridných vzťahov boli použité maternálne dedené markéry, konkrétne 6 chloroplastových

mikrosatelitov, ktoré všetky vykazovali polymorfizmus. V analyzovanom materiáli sme našli 6 haplotypových kombinácií. Haplotyp 2 bol nájdený len u *S. chamaemespilus*, populácie tohto taxónu boli monomorfné. Haplotypy 1 a 4 sa našli len u *S. aria*, haplotypy 3 and 5 sme identifikovali u *S. aria* a *S. haljamovae*. Vzorka *S. aucuparia* použitá vo fylogenetickej analýze pre zakorenenie fylogramu, vykazovala špecifický haplotyp výrazne divergentný od zostávajúcich (obr. 1). Jedine populácie *S. aria* boli polymorfné. Na lokalitách so spoločným výskytom *S. aria* a *S. haljamovae* oba taxóny vždy zdieľali chloroplastový haplotyp (obr. 2). Tieto výsledky naznačujú, že *S. haljamovae* je polyfyletický taxón, ktorý vznikol prinajmenšom dvomi nezávislými kríženiami rodičovských druhov a následne sa rozšíril na ďalšie lokality.

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