# Pollen morphology of Norwegian hybrids of Sorbus L.

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#### Abstract

The genus Sorbus consists of small to medium-sized trees primarily found in coastal areas, with three European hotspots for diversification: Fennoscandia, South-East Europe, and Britain. Taxonomic classification of Sorbus in Europe is still unresolved, and this study aims to address this issue by examining the pollen morphology of different Sorbus hybrids. Pollen morphology of 16 specimens from 13 species of Sorbus, Aria and Hedlundia, some of which are common, while others are endemic and listed as threatened species, were studied. Measurements of the polar and equatorial axis of all pollen show that there are variations in pollen size and shape among different hybrids, with correlation between polyploidy levels and the width of the pollen. Surface structures of pollen were observed using SEM. Principal Component Analysis (PCA) was performed to analyze the pollen characters, and the results show that it is possible to separate Aria from Sorbus and Hedlundia. The study concludes that pollen morphology can be a useful tool for distinguishing between different Sorbus hybrids and can thus aid in understanding their past distributions.

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#### **Keywords**

Rosaceae, polyploidy, pollen surface structure, pollen size, taxonomy

#### Introduction

Species of *Sorbus* L. are small to medium sized trees abundant in coastal areas, with three European hotspots for continued *Sorbus* diversification and speciation: Fennoscandia, South-East Europe, and Britain (Robertson et al., 2010).

Sorbus is an agamic species complex, resulting from hybridization, polyploidy and apomixis (Hörandl, 1998), where interspecific hybridization, the main resource of diversity in *Sorbus*, is usually followed by apomictic reproduction. Due to rapid evolution, speciation, and hybridization, multiple speciation events have occurred in parallel within different lineages of *Sorbus* (Li et al., 2017). The study of *Sorbus* faces several complex taxonomic issues that have yet to be resolved in Europe (Sennikov and Kurtto, 2017). Hybridization between species with differing chromosome numbers followed by polyploidization and the production of seed sterile offspring are propagated and stabilized as new taxa by apomixis.

The genus *Sorbus* belongs to the apple tribe Maleae in the Rosaceae family and in the traditional sense used to include more than 100 species widespread in the Northern Hemisphere according to POWO (2022). Hybridogenous taxa of *Sorbus* have been commonly left unidentified, until Sennikov and Kurtto (2017) assigned a new intrageneric nomenclature for the nothotaxa. Hybridogenous species are mostly regarded as species in the same way as non-hybrid species, whereas causal hybrids are not equivalent to species (Baker and Bradley, 2006). Recently, several new genera were established for all hybrid taxa between *Aria*, *Sorbus*, *Torminalis*, and *Chamaemespilus* by Sennikov and Kurtto (2017). The majority of the Norwegian species are hybrids between *Aria* and *Sorbus*, hence treated under the new genus name *Hedlundia*. This approach is still under debate (Levin et al., 2018), but obviously *Sorbus* has five main evolutionary branches and is polyphyletic (Lo and Donoghue, 2012; Tang et al., 2022).

Currently, 13 species of *Sorbus* in a wide sense including *Sorbus* hybrids are registered in Norway. Some of these taxa are very common, and others are endemic and listed as threatened species (IUCN, 2022). Taxonomical challenges within *Sorbus* have been discussed amongst Nordic botanists for decades, starting with Hedlund (1901) publishing the first comprehensive monograph describing the variation in *Sorbus* species. Later Liljefors (1955) hypothesized on how the Nordic taxa of *Sorbus* were related, and how the sexual diploids at the base of the lineages *Sorbus*, *Aria*, *Torminalis*, and *Chamaemespilus* have been hybridizing, resulting in triploid and tetraploid taxa, forming the backbone of the origin of the polyploid endemics.

The base chromosome number of *Sorbus* in a wide sense is x=17. Diploid, triploid, and tetraploid members are respectively 2n=2x=34, 2n=3x=51 and 2n=4x=68 (Pellicer et al., 2012).

The five hybridogenous triploid species of Hedlundia (H. subarranensis , H. sognensis , H. subpinnata , H. neglecta , and H. lancifolia ) have chromosome numbers 2n=51, with semipinnated simple leaves (Sennikov et al., 2016). H. subarranensis has a very limited distribution in western Norway, endemic to Norway, and is listed as endangered by IUCN. H. sognensis is even more rare, endemic to also western Norway, and listed as endangered, as H. subpinnata , endemic and limited to south-east Norway. H. neglecta and H. lancifolia are found in northern Norway and are both endemic. H. neglecta is listed as endangered by IUCN, while H. lancifolia is critically endangered.

Tetraploid hybridogenous species with 2n=4x=68 chromosomes of this complex include *H. meinichii*, *H. subsimilis*, *H. hybrida*, *H. mougeotii*, *A. obtusifolia* and *A. rupicola*. The four species *H. subsimilis*, *H. mougeotii*, *A. obtusifolia* and *A. rupicola* have simple leaves, while *H. meinichii* and *H. hybrida* have compound leaves. *H. meinichii* grows in coastal areas in southern Norway and is endemic to Norway. *H. meinichii* is very variable, including both triploids and tetraploids, and several different populations containing distinct morphs (Salvesen, 2011). It is listed as vulnerable by IUCN but is unsolved due to undescribed variation. *H. subsimilis* is distributed in southern parts of Norway, is endemic to this area, and listed as vulnerable by IUCN. *H. hybrida* is common along the coast in Norway and is as *H. meinichii* a variable species with several morphs. *H. mougeotii* is widespread in Europe, but scattered in Norway, as it is an introduced species. *A. obtusifolia* is distributed along the coast of southern Norway with a few finds just across the Swedish border and is endemic to Scandinavia.*A. rupicola* has a wider distribution in northwest Europe and is found scattered along the coast to northern Norway.

Morphological traits of leaves and fruits have for a long time been used to classify hybridogenous *Sorbus* taxa (Hedlund, 1901). The shape of leaves has proven useful to differentiate these taxa to genus and species level.

On the extremes, *Sorbus aucuparia* (2n) has compound leaves divided into 5-8 pairs of serrated leaflets and is distributed all over Norway as a very common species, while *Aria edulis* (2n), *Aria rupicola* (4n), and *Aria obtusifolia*(4n) have simple leaves and a limited distribution in Norway, the former only as naturalized from gardens. All species of *Hedlundia* presented here have leaves of a shape intermediate between these opposite types.

Sorbus pollen morphology is diverse, and there is significant variation at the intra and interspecies level (Boyd and Dickson, 1987). Using only a light microscope (LM) the separation of pollen from species within the genus is difficult, as *Sorbus* pollen is very similar to other taxa of the Rosaceae family (Boyd and Dickson, 1987; Eide, 1981; Reitsma, 1966). Most studies of fossil pollen have not identified *Sorbus* pollen to lower taxonomic levels than *Sorbus* -type or Rosaceae. *Sorbus* pollen is tricolporate, psilate or intrastriate, and suboblate to subprolate with maximum length of 23-42  $\mu$ m (Boyd and Dickson, 1987). Gabrielian (1978) separated diploid species of *Sorbus* from Western Asia and the Himalayas based on shape and size of pollen, type and number of apertures, and thickness and structure of the exine. Using scanning electron microscope (SEM), identifications can be done to a lower taxonomic level than if you use light microscope (LM), as Bednorz et al. (2005) has shown to study the Polish species of *Sorbus*. Bednorz et al. (2005) describes *Sorbus* pollen as belonging to the non-operculate group as it lacks an aperture membrane, and the differences in costa colpi are the most important feature. Despite few studies on the pollen morphology of *Sorbus* species, little is known in general about the Norwegian hybridogenous taxa and their pollen morphology.

The main aim of this paper is to describe the variation in pollen morphology and to investigate whether this is a useful tool to separate between pollen from different Norwegian hybrids of *Sorbus*. This will make it possible to study the past distributions of *Sorbus*hybrids.

#### Material and methods

Specimens were collected from various parts of Norway in 2019, either from native stands or from cultivated plants of known origin (Figure 1, Table1). Inflorescences were collected and dried for studies of pollen morphology, and herbarium specimens were sampled and prepared for documentation, deposited in herbarium BG.

DNA ploidy levels were measured for the studied specimens except samples of *A. rupicola*, *H. lancifolia*, *H. neglecta*, *H. subpinnata*, and *S. aucuparia* (Table 2). The ploidy levels were measured using flow cytometry analysed by Plant Cytometry Services (Schijndel, The Netherlands) following the procedure given in Andersen et al. (2016).

Anthers were treated with 10% KOH to remove cell content, followed by acetolysis to remove cellulose (Fægri et al., 1989). The pollen was stained using fuchsine and mounted in glycerol. Using light microscope (LM) the polar axis (P) and the equatorial axis (E) of 100 pollen grains of each specimen were measured. The P/E ratio was calculated following Boyd and Dickson (1987). In total 1600 pollen from 16 different specimens were measured. To assess and compare the shape, central tendency, variability of the samples, and to look for outliers in 16 different specimens, the side-by-side boxplots were made of the lengths of the polar axis (P), equatorial axis (E), and the calculated ratio (P/E) of the 100 measured pollen for each specimen (Figure 2).

Scanning Electron Microscope (SEM) was used to investigate the surface structure of the pollen and to aid the description of pollen of the specimens. Pollen was applied with a brush, carefully sprinkled over carbon tape, and glued to an aluminium rivet. Following by coating with a thin layer of gold and palladium to make the sample electrically conductive throughout. Individual pollen grains were visualised with a Zeiss Supra 55VP scanning electron microscope with 5.00 kV, 30  $\mu$ m aperture with a working distance to the lens ~4 mm. From the resulting pictures, morphological characteristics of the pollen surface were recorded (Table 3).

Principal Component Analysis (PCA) was first run with the three measures pollen characters, the polar axis (P), equatorial (E) axis, and their ratio (P/E). A second analysis with the nine different characters of pollen morphology; including the measured characters and the morphological characters of the pollen

surface as given in Table 3. The analyses were performed with centring and standardization of characters and visualized in CANOCO 5 (Ter Braak and Šmilauer, 2012).

#### Results

The results from the flow cytometry are given in Table 2, together with the inferred ploidy levels, showing that the *Sorbus* hybrids (*Hedlundia* or *Aria*) are either triploids or tetraploids.

Size differences in length of the equatorial axis (E) of the measured pollen are shown as boxplot in Figure 2. The mean values vary from  $12 - 19.5 \,\mu\text{m}$ . The longest E value was measured in *A. obtusifolia* and shortest equatorial axis is found in *H. neglecta*. *S. aucuparia* and *A. obtusifolia* pollen show the least and most variation, respectively (Figure 2). Comparing the length of E and polyploidy level of the specimens there is a clear correlation between the diploid, triploid, and tetraploid specimens, and length of the equatorial axis (Figure 2).

Measurements of the polar axis (P) are given as boxplots in Figure 3 and show that it is *H. neglecta* that has the shortest polar axis, and *A. edulis*, *A. obtusifolia* and *H. hybrida* 1 have the longest. The measured means of all specimens vary between 13 and 26  $\mu$ m. The polar length in *H. hybrida* 1 grain shows most variation within the measurements. Many specimens have a P between 16 and 20  $\mu$ m. When comparing the length of P and polyploidy level of the specimens, there is no correlation between the diploid, triploid, and tetraploid specimens, and length of their equatorial or polar axes. But, except for *H. hybrida* 1, *Aria* has larger pollen than the other samples tested.

According to Erdtman (1952) the pollen shape can be categorized based on the P/E ratio, and her separates pollen as oblate-spheroidal if the ratio is 0.89-0.99, spheroidal when P/E is 1.00, prolate-spheroidal between 1.01 and 1.14, sub-prolate between 1.15 and 1.33, and prolate if the ratio is higher than 1.34 (Figure 4). The pollen measured in this study all have a mean ratio > 1.00. Following this we see that *H. subsimilis*, *H. meinichii* 1+2, *H. subpinnata*1+2, *H. mougeotii*, *H. neglecta*, *A. rupicola*, and *H. subarranensis* have a mean ratio between 1.01 and 1.14 and are prolate-spheroidal. *H. sognensis*, *H. lancifolia*, *H. hybrida* 2, and *A. obtusifolia* have a mean ratio in the sub-prolate group, and *H. hybrida* 1, *S. aucuparia* and *A. edulis* are prolate.

In Figure 4, the ratios are compared to the polyploidy level of the specimens. The two diploid species, A. *edulis* and S. *aucuparia* have the largest P/E ratio in this study. The tetraploid H. *hybrida* 1 has significantly larger pollen compared to the other triploid and tetraploid specimens.

Details of the surface of the pollen grains are not always clear while observing through light microscope (LM), and for that reason, scanning electron microscopy (SEM) was used to observe the surface structures and details of the specimens. Selected images of all our studied specimens are given in Figure 5, showing the polar view, the equatorial view, and details of the surface pattern. The morphological characters of the surface and the corresponding pollen shape for our study specimens are listed in Table 3. Most visible is the long colpi in all our studied specimens, directions and length of striae, number of perforations, and the shape of the pollen grain in polar view.

The PCA ordination for the pollen measures of P and E, and their ratio is given in Figure 6, where 99.91 % of the variation is explained by the two first axes. The diploid *S. aucuparia* and *A. edulis* have a clearly higher ratio, as also seen in Figure 4. The tetraploid specimens have a higher value of both P and E, compared to the triploid specimens, but overlapping.

The results from the PCA ordination analysis of all the pollen characters are shown in Figure 7, where 58.68 % of the variation is explained by the two first axes. The diploid species *S. aucuparia A. edulis*, have higher values for polar length (P), as also seen in Figure 6, in addition to pollen perforation. The specimens are grouped by genus in this figure and shows that *Aria* has larger values of the measured characters and are grouped separately from *Hedlundia*. *Hedlundia* on the other hand, has large variation between the different specimens, with the extremes *H. hybrida* 1, *H. lancifolia*, and *H. neglecta*.

### Discussion

Pollen morphology of species of *Sorbus* s.lat., *Aria* and their hybrids in Norway (*Hedlundia*), is rarely studied in detail. The first European studies of pollen morphology of *Sorbus* in general, was Erdtman (1952) and Praglowski (1963), later followed by Eide (1981). These studies were novel as they believed that *Sorbus* pollen could be identified, and not only categorized as *Crataegus* -type of pollen as earlier. Later, Boyd and Dickson (1987) studied pollen of *S. aucuparia* (2n), *A. rupicola*(4n), *H. arranensis* (3n), and *H. pseudofennica* (4n), using LM. They found that *S. aucuparia* could be separated from the other taxa but found less differences between the hybrid species. Bednorz et al. (2003) found that while size measures were overlapping for species and hybrids of *Sorbus*, exine sculpturing provided features for taxonomic classifications. There has been a general opinion amongst palynologists that pollen from the family Rosaceae only is useful for morphological identification on higher ranks, like subfamily or genus. Wronska-Pilarek et al. (2022) points out that studies report useful morphological characters for species delimitation in the Rosaceae, such as the length of the polar axis (P), pollen shape (P/E), operculum structure, and presence/lack of costae colpi.

Bednorz et al. (2005) concludes that "... it is relatively easy to separate pollen grains" of the genera *Sorbus*, *Chamaemespilus*, and *Torminalis* using SEM. They found that type of sculpture, length of polar axis (P), number of ectocolpi, and ratio of the ectexine to endexine thickness are the most important separating characters for these genera. These taxa were previously defined within the genus *Sorbus* but are now classified as genera (Sennikov and Kurtto, 2017).

Our results show that pollen of Aria generally has a longer polar axis (P) than pollen of Sorbus and Hedlundia (Fig. 3). From the PCA analysis (Fig. 7), Aria has clearly larger pollen size in general, compared to Sorbus and Hedlundia . As Aria and Sorbus are not closely related (Lo and Donoghue, 2012), but Aria is related to Malus , whereas Sorbus related to Pyrus , it is expected to see differences between these genera, and the main separating character is size, and more specific, the length of the longest axis (P). For the hybrids (Hedlundia ), it has been hypothesized that Sorbus is the ovule donor (Liljefors, 1955), and therefore it's reasonable to believe that pollen mostly resembles their mother in size.

Pollen size was categorized in different intervals by Erdtman (1969). Pollen with polar axis (P) between 50-100  $\mu$ m are large, 25-50  $\mu$ m are medium sized, and between 10-25  $\mu$ m are defined as small. From our measurements, *A. edulis*, *H. hybrida* 1 and *A. obtusifolia* have a mean P value above 25  $\mu$ m and are therefore medium sized. All other specimens in our study have small pollen. With its medium sized pollen, *H. hybrida* 1, stands out as very large compared to all other species of *Hedlundia*. *H. hybrida* is a morphologically very variable species, and different subspecies have been suggested (Grundt and Salvesen, 2011). This variation is also seen here, when comparing the two different specimens of *H. hybrida* in our study. In the other end of the range are *H. neglecta*, with very tiny pollen.

We have compared our measurements of the shape of pollen (ratio P/E) with other available studies and see that there is a large range between studies (see Figure 8). In general, our samples have a higher ratio than in all the other studies, whereas Eide (1981) has lower ratios than the others. This can be related to different preparation techniques. According to Katsiotis and Forsberg (1995) pollen size can be influenced by treatment methods and mounting media. Pollen grains tend to swell in glycerol (Moore and Webb, 1978). This can lead to different sizes in different studies and can explain why our measured pollen has a higher ratio as the samples are all mounted in glycerol.

S. aucuparia pollen ranges from a ratio of 0.96 in Eide (1981) to 1.48 in this study (Figure 8). This gives rise to all forms of pollen; oblate spheroidal form (Eide, 1981), prolate-spherioidal shape (Bednorz et al., 2003; Boyd and Dickson, 1987; Erdtman et al., 1961), subprolate shape (Bednorz et al., 2005; Praglowski, 1963), and prolate shape in this study. S. aucuparia is a morphological extreme variable species, and many have suggested establishing subspecies to account for this variation. This may also be reflected through this study of pollen shape.

For *H. hybrida*, the variation in P/E is lower, ranging from 1.05 to 1.36, but still giving rise to three different shape forms. *H. hybrida* is also a very variable species, resulting from hybridization events with different

parent trees. Our two samples of H. hybrida originates from different parts of Norway, H. hybrida 1 from Telemark (4), eastern Norway, whereas H. hybrida 2 is collected in Sogndal (5) in western Norway and is a morphological very different type. From the comparison of different data on the shape of Sorbus pollen, we can conclude that shape is only useful where the methodology is consistent.

Erdtman (1952) hypothesized that pollen size is dependent on polyploidy. Erdtman (1969) writes that "Pollen grains and spores in polyploid species are, as a rule, larger than in diploids". In general, it is argued that larger amount of DNA per cell will make larger cells, both for pollen and spores. Several examples of species with larger pollen size correlated with ploidy levels are shown, as for *Andropogon*by Gould (1957), for *Avena* species studied by Katsiotis and Forsberg (1995), and Altmann et al. (1994) even used pollen size to determine the ploidy level of *Arabidopsis thaliana*.

After the "Eukaryotic genome size databases" (Gregory et al., 2007) was launched, Knight et al. (2010) used this database together with pollen size data from different sources to study the relation between pollen and genome size. They found that from 464 species, there was a positive correlation between pollen width (E) and genome size. Based on literature, Knight et al. (2010) estimated that pollen size increase by 1.1x-2x with double C-value (amount of DNA in a haploid genome).

When plotting our mean pollen width (E) against ploidy level (DNA amount) we find a positive correlation of 0.45, as shown in Figure 2 (small box). This supports the hypothesis that more DNA results in larger pollen, hence pollen of polyploids are thicker than diploids.

Eide (1981) argued that exine ornamentation was one of the most important features not only to identify the pollen of *Sorbus*, but also the Rosaceae. Bednorz et al. (2003) finds that the exine sculpturing was the main character for distinguishing pollen from *S. aucuparia*, *H. arranensis*, *H. hybrida*, and *H. mougeotii*, and concluded that *S. arranensis* was distinct from the other species by its short rugulate-like exine pattern. Both *S. hybrida* and *S. mougeotii* were characterized by very long striation, with the striae clearly thinner at *S. mougeotii*. The *S. aucuparia* pollen had shorter striae and dense, well visible perforations between vallae.

The variation in ornamentation of our samples is shown in the SEM pictures given in Figure 5. The variation in surface structures is clearly visible and a basis for classification of the taxa included in this study. Perforations is variable, and a basis of our key to identify Norwegian species:

Medium sized pollen (25-50  $\mu$ m)

Few perforations Aria obtusifolia

Medium perforations Aria edulis

Dense perforations Aria rupicola

Small pollen (10-25  $\mu$ m)

Few perforations

Polar view circular Medium length of striae *Hedlundia sognensis*Short striae *Hedlundia subpinnata* Polar view triangular *Hedlundia neglecta* 

Medium perforations

Short striae Hedlundia hybridaLong striae Hedlundia mougeotii

Dense perforations

Circular polar view Short striae in parallel directions *Hedlundia subarranensis* Medium striae in variable directions *Hedlundia subsimilis*Long striae in parallel directions *Sorbus aucuparia* Triangular polar view Striate-rugulate exine *Hedlundia lancifolia*Striate exine *Hedlundia meinichii* 

It is possible to identify pollen of *Sorbus*, *Aria* and *Hedlundia* with morphological characters using LM only, but using SEM will ease the identification. The identification will need a very close study of the given characters but will be feasible.

This study of pollen morphology of *Sorbus* and *Aria* with their hybrids (*Hedlundia*), shows that the genus *Aria* has longer pollen than both *Sorbus* and *Hedlundia*, and that these are separated by size. *Sorbus* pollen is smaller than *Hedlundia* but with some overlap. By using size in combination with several characters of surface ornamentation, hybrids are possible to identify by pollen morphology.

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Figure 1. Map of Norway showing the positions sample collection areas. Numbers correspond to numbers given in Table 1.

Figure 2. Box plot of the measured equatorial width (E) of pollen. The box encloses the middle half of the data of the second and third quartiles, the thick horizontal line is the median, and the vertical lines show the range of data values in the first and fourth quartiles. Extreme outliers are shown as open circles. Colors of boxes refer to ploidy level (from Table 2). The inset box shows a plot of the correlation between E and ploidy level. The correlation is 0.45.

Figure 3. Box plot of the measured polar length (P) of pollen. The box encloses the middle half of the data of the second and third quartiles, the thick horizontal line is the median, and the vertical lines show the range of data values in the first and fourth quartiles. Extreme outliers are shown as open circles. Colors of boxes refer to ploidy level (from Table 2).

Figure 4. Box plot of the measured of the P/E ratio. The box encloses the middle half of the data of the second and third quartiles, the thick horizontal line is the median, and the vertical lines show the range of data values in the first and fourth quartiles. Extreme outliers are shown as open circles. Colors of boxes refer to ploidy level (from Table 2) and vertical dotted lines mark different shapes of pollen following Erdtman (1952).

Figure 5. Scanning electron microscopy (SEM) images pollen from each specimen analysed in (1) polar view, (2) equatorial view, and (3) close-up of pollen grain exine ornamentation. The scale bars are 2 µm.

Figure 6. The first two axes resulting from the Principal Component Analysis (PCA) of pollen measurements (P and E) and the calculated ratio (P/E) from this study. The ploidy levels (from Table 2) are referred to in the different colors.

Figure 7. The first two resulting axes from the Principal Component Analysis (PCA) of all pollen characters (as given in Table 3). Different genera are marked in colors.

Figure 8. The calculated P/E ratio from measurements in this study compared with several other studies. Colors of bars refer to the data source.

**Table 1.** Species included in this study with origin, herbarium, and collection numbers, date of collection, and endemism status. Numbers in brackets are referring to Figure 1.

Table 2. DNA content with measured or given polyploidy levels. \* are taken from (Kurtto et al., 2018).

Table 3. Morphological characteristics of the pollen surface interpreted from the SEM pictures in Figure 5.

















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