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Selection of source material for introduction of the locally rare and threatened fern species *Asplenium septentrionale*

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**Background:** Forked spleenwort, *Asplenium septentrionale*, is a mainly petrophilous fern species in European mountains and rare on acidic siliceous rocks in lowland areas of the continent, where habitats are fragmented and populations isolated. In Estonia, the single extant population is very small, occupies a restricted area and is threatened by human disturbances. An introduction project of the species was prepared to form new populations in new protected sites using *ex-situ* propagated young sporophytes as transplantation material.

**Aims:** To obtain data on the species recruitment population biology and provide context information for selecting donor plant material.

**Methods:** We sampled three regional/local donor populations. First, we carried out a laboratory breeding experiment to evaluate the populations’ ability for intra-gametophytic selfing. Second, to estimate differences in fitness of offspring among the populations, we grew young sporophyte plants in a pot experiment under controlled conditions in a common garden.

**Results:** The Estonian population showed very high capacity (90%) for intra-gametophytic selfing, as well as high rate of sporophytic mortality (83%), but the rates are comparable to one of the reference populations in Finland. However, plants of Estonian population were smaller.

**Conclusions:** The Estonian population may represent a unique pre-adapted or locally adapted genotype; therefore, it needs more efficient protection in its present location. Planting material for introduction should be collected from the local population, as the best locally adapted. Only in the risk of severe environmental change and of extinction, several neighbouring populations could be pooled to maximise genetic diversity.

**Keywords:** *Asplenium septentrionale*; conservation planning; intra-gametophytic selfing; plant conservation; plant introduction

**Introduction**

Two different groups of species-specific factors need to be considered when planning plant species (re)introduction – those associated with the biology and ecology of the species, and those related to socio-economic and legal requirements (International Union for Conservation of Nature 1998). In this paper, we use the term ‘introduction’ as a stricter term than ‘reintroduction’ (i.e. any controlled placement of plant material into a natural or managed ecological area, Godefroid et al. 2011), to refer to planting of *Asplenium septentrionale* *ex-situ* propagated sporophytes within their normal range, but in areas (sites) where it is not presently known (Allen 1994).

The selection of appropriate new habitats and suitable donor plant material are equally important (Falk et al. 1996; Kaye 2008; Maschinski et al. 2012). The specific strategy for reintroduction depends primarily on the specific goals and objectives and may differ when establishing new populations in old, previously occupied (extinct) habitats (Fiedler and Laven 1996; Maschinski and Duquesnel 2006), or when introducing new populations in previously unoccupied habitats (Aleric and Kirkman 2005; Maschinski and Wright 2006). Nevertheless, the choice of donor plant material used to create new populations is critical to the success of any translocation project (Guerrant 1996; Godefroid et al. 2011).

Different specific aspects of species recruitment biology and a population’s genetic structure should be considered when selecting suitable donor plant material for (re)introduction (Falk et al. 2006; Maschinski et al. 2012; Neale 2012), starting with the ability of lightweight microscopic spores to be dispersed over long distances by winds and to occupy specific fragmented habitats. The probability of a single spore colonisation is determined by the ability of species for intra-gametophytic selfing (inbreeding) – a mode of sexual reproduction in which both gametes originate from the same gametophyte. Although inbreeding produces completely homozygous sporophytes, spores itself are not identical because of recombination during their formation. Also, polyploid species are functionally heterozygous at each locus because of duplicated genes, and exhibit low levels of genetic load (Otto and Marks 1996; Haufler 2007). Simple breeding experiments in which single gametophytes are grown in isolation have been frequently used to evaluate populations’ capacity for intra-gametophytic selfing and the level of population genetic load (Ranker and Geiger 2008). The ability for intra-gametophytic selfing can be quantified as the percentage of bisexual gametophytes that produce sporophytes and the

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level of genetic load as the percentage of bisexual gametophytes that do not produce sporophytes (Peck et al. 1990).

Planning the establishment of a new population requires consideration of the genetic characteristics of the type of donor material (Falk et al. 2006; Maschinski et al. 2012; Neale 2012). Different local populations are likely to be affected by different environmental factors and as a result distinct locally adapted genotypes (ecotypes, Hufford and Mazer 2003) are expected to arise within a species (Turesson 1922; Linhart and Grant 1996). Naturally, a mixture of genotypes from similar but spatially distinct donor environments may be the best option to maximise the probability that at least some surviving genotypes would be included (Montalvo and Ellstrand 2000; Hufford et al. 2008; Bennington et al. 2012). In addition, genetically variable donor material could increase the resilience of the newly established population to possible future environmental changes (Lesica and Allendorf 1999; Gray 2002; Menge 2008; Neale 2012).

The easiest and probably the cheapest method to study genetic differences among local populations is to grow plants from different populations under controlled conditions, such as in a common garden. In a uniform environment, differences in morphological and/or life-history components of fitness among different populations may reveal underlying genetic differences among populations (Clausen et al. 1948). Although results from common garden experiments do not directly reveal local adaptations of specific populations or origins of genetic variation (natural selection or genetic drift, Hufford and Mazer 2003), common garden experiments can still shed light on the genetic diversity in populations and provide guidelines for introduction projects and future research.

In Estonia, we have the introduction project of *A. septentrionale* underway. The only extant Estonian population of *A. septentrionale* inhabits a very small area in an anthropogenic habitat, subjected to trampling and other disturbance. The aim of the introduction project is to form new population(s) of the species in protected public land free from direct negative human impact on the same island where the extant population is found, and possibly on the mainland in Northern Estonia. Rünk et al. (2014) reported detailed data on the ecology of *A. septentrionale* and provided information for selecting suitable introduction sites. Present study forms the second part of this introduction project.

The objective of this study was to obtain detailed data on the recruitment biology of the species and to provide information for selecting suitable donor material for introduction. We carried out two experiments – a breeding experiment to estimate the capacity of the extant population for intra-gametophytic selfing (Experiment 1); and a common garden experiment to examine sporophyte mortality (Experiment 2.1) and possible local morphological adaption of the Estonian population (Experiment 2.2.). More specifically, we hypothesised firstly that the Estonian population of *A. septentrionale* exhibit a high rate (> 60%) of intra-gametophytic selfing (Aragón and Pangua 2003). Secondly, we expected variation in plant fitness between populations grown in a common garden, specifically that plants that originated from the Estonian population of *A. septentrionale* exhibited lower fitness than plants originating from the geographically nearest large set of populations in Finland.

**Material and methods**

**Study species**

The sporophyte of *A. septentrionale* is a low to short-growing rhizomatous herbaceous perennial with simple or dichotomously 1–3 times forked linear lamina (Crabbe et al. 1993; Wagner et al. 1993). The short rhizome of a plant has short densely branches, and the genet forms a compact, grass-like clump with wintergreen leaves. *A. septentrionale* is a sexual autotetraploid (2n = 144) species derived from the diploid cytotype (referred as *A. septentrionale* subsp. *caucasicum* Fraser-Jenkins & Lovis, Lovis 1964).

*A. septentrionale* is distributed in Europe, Asia and North America (Hultén and Fries 1986, Figure 1), mainly in rocky habitats (Crabbe et al. 1993; Wagner et al. 1993). Although in Europe the species is fairly common in the mountainous areas, it is absent or rare with disjunct and isolated populations in lowland areas, particularly in the eastern, Atlantic and Mediterranean regions (Jalas and Suominen 1972). In the British Isles, the species has been included in The Vascular Plant Red Data List for Great Britain (Cheffings and Farrell 2005), in the category of “near threatened”, it is scarce (Hill et al. 2004), very local (Page 1997) and showing declining trend in the distribution (Rumsey 2002). In Denmark, *A. septentrionale* is very scattered and declining (Øvstedal and Tiggerschiod 2000); in Netherlands, the species considered as a very rare vagrant species (FLORON 2014) and in Flanders (northern Belgium), critically endangered (CR) (Van Landuyt et al. 2006). The Estonian Red List of Threatened Species 2008 classifies *A. septentrionale* as CR (eBiodiversity 2008); and it is strictly protected in the country. The only Estonian extant population is located at one small island in the Gulf of Finland, at the south-eastern edge of the Fennoscandian part of its distribution range.

**Experiments**

**Experiment 1.** Spores were collected from three populations – from the single Estonian population and two populations around Helsinki (Western Helsinki and Eastern Helsinki), Finland in August 2007. The distance between Estonian and both Finnish populations is ca. 70 km and between the two Finnish populations ca. 10 km. These Finnish populations are the closest to the Estonian only population and probable sources of plant material (spores for propagation) for reinforcement plantings and introduction. Spores were collected from seven randomly chosen plants in each population and mixed within the populations. Spores were sown in 9-cm plastic Petri dishes on 9 October 2007. The substrate for spore germination (pH = 4.9) was sterilised and consisted of two parts of horticultural peat, one part of moderately white peat
moderately decomposed remains of mainly different species of *Sphagnum* spp.) and one part of fine-grade sand. The Petri dishes were placed in the laboratory at 20 ± 2 °C with a photoperiod of 12 h (fluorescent light: daylight tubes, photon flux density 40 µmol m⁻² s⁻¹). After 5 weeks, 60 randomly selected filamentous gametophytes from each population, still in pre-sexual phase (as suggested by Aragón and Pangua 2003), were transplanted as isolated specimens onto fresh substrate in new 9-cm Petri dishes. Even if each Petri dish contained four gametophytes, they were isolated by plastic walls, ensuring that all gametophytes will have intra-gametophytic selfing and could not have inter-gametophytic selfing or outcrossing. Petri dishes were randomly rearranged weekly, all gametophytes watered with distilled water and examined for the presence of sporophytes. The appearance of sporophytes was recorded. The first sporophyte emerged 131 days and the last 263 days after sowing.

**Experiment 2.** The objective of this experiment was to evaluate the fitness of young sporophytes for introduction. The experiment consisted of two subphases, the experiment 2.1 concentrated on sporophyte mortality rate during ca 3–4 years, and the experiment 2.2 evaluated fitness as plant leaf properties. Plants, sown in 9 October 2007, were grown with mixed set of gametophytes from different parent plants so that selfing and crossing was possible within each population, just as it would appear in nature within isolated populations. On 15 October 2008, plants were relocated into an unheated polyethylene greenhouse where they were grown to the end of the experiment, where plants wintered covered with white peat as a proxy for fallen leaves and their decayed remnants. In the growth periods (from April/May–October/November), plants were inspected at 1–3-day intervals, depending on weather conditions, and watered as needed to keep the soil moist. In order to minimise differences in illumination conditions, the positions of the pots were changed weekly. Gametophytes and emerged sporophytes were grown until May 2009 in plastic boxes (15–50 individuals evenly spaced per box) on the same substrate and in the same laboratory as in Experiment 1. Then, they were transplanted several times into boxes with increasing size. At the start of new vegetation season, on 15 May 2009, 210 young sporophytes (count varied among populations) were planted into individual plastic pots (6 × 6 × 6 cm), and replanted on 7 May 2010 into fresh substrate of the same composition and pots of the same size. On 23 May 2011, all plants were again replanted into fresh soil, but into larger pots (7 × 7 × 7 cm).

In October 2011, the number of leaves was counted and the length of the longest leaf was measured on 12 randomly chosen individuals of each population. All plants with leaves were generative with sporophylls. The number of sporophylls was not counted to avoid disturbance to the fragile and dense clusters of leaves.

**Statistical analyses**

**Experiment 1.** Differences among three populations in the success rate to form sporophytes after intra-gametophytic selfing event on transplanted and spatially isolated filamentous

Figure 1. The circumpolar distribution of *Asplenium septentrionale*. Reproduced from Hultén and Fries (1986) Atlas of North European Vascular Plants North of the Tropic of Cancer, Volume I, by permission of Koeltz Scientific Books, Königstein, Germany. The main distribution area of *A. septentrionale* is hatched; restricted areas are marked with black circles (●).
gametophytes were tested using analysis of generalised linear models with setting of binomial error distribution and logit link function, implemented in programme Statistica ver 9 (StatSoft 2004). We used binary response, i.e. either the sporophyte on filamentous gametophyte was formed or not. Pairwise differences among single populations were estimated using contrast of parameter estimates within the model. Probability estimates of formation were retransformed from logit-transformed model prediction. Therefore, 95% confidence intervals around means as asymmetric.

Experiment 2.1. Differences in sporophyte mortality among plants of different origin populations (Estonia, Western Helsinki and Eastern Helsinki) were analysed using generalised linear models with same settings as in Experiment 1.

Experiment 2.2. The difference in morphological traits of plants with different origin population. We tested for differences in number of leaves and length of the longest leaf (plant height) on 12 plants of each population using one-way ANOVA (StatSoft 2004). All variables were log transformed before the analysis to meet the assumptions of ANOVA (Zar 1999). Tukey HSD multiple comparison test with 0.05 significance level was used to detect significant differences in morphological traits among the plants of different origin (Sokal and Rohlf 1995).

Results

Of the isolated *A. septentrionale* gametophytes for intra-gametophytic selfing, the probability to produce sporophyte varied significantly among populations ($\chi^2_{df=2}=20.44; P=0.00004$). The proportion of produced sporophytes (Figure 2) was the lowest in the population from the Western Helsinki – 56.7% of the gametophytes formed a sporophyte. There was no significant difference between the formation of sporophytes in populations from Estonia and Eastern Helsinki – 90.0% and 86.7%, respectively.

After 4 years of the experiment 2.1, the mortality of *A. septentrionale* plants differed between populations ($\chi^2_{df=2}=17.15; P=0.00019$; Figure 3). Plants from Estonian and Eastern Helsinki populations had much higher mortality rate (83.3% and 77.8%, respectively) than plants of Western Helsinki population (45.2%).

In Experiment 2.2, plants from Estonian population were smaller than both Finnish populations (Figure 4). Specifically, number of leaves differed significantly among populations ($F_{2,33}=5.713; P=0.0074$), but the main difference was among Estonian population and Western Helsinki population. Plants from both Finnish populations had larger length of the longest leaf than those from the Estonian population ($F_{2,33}=8.22; P=0.0013$).

Discussion

The high mean percentage of sporophyte production after intra-gametophytic selfing (Estonian population 90.0%) *A. septentrionale* exceeded the expectation of 63% obtained by Aragón and Pangua (2003) for gametophytes originating from Spain. Differences in sporophyte production can first of all be attributed to different sex expression (Cousens 1988; Aragón and Pangua 2003) – apparently not all gametophytes became bisexual and were able to produce sporophytes. Inability of producing sporophytes could also be explained also by other factors, e.g. the abnormal development of gametangia, absence or deficiency of sperms, or by inbreeding depression (Suter et al. 2000). The effect of an environmental factor (the lack of water needed for fertilisation) in controlled laboratory conditions is unlikely, but not impossible.

The high percentage of sporophyte production in isolated gametophytes indicates a high capacity for intra-gametophytic selfing and therefore a high capability of single-spore colonisation. The existence of a high...
number of genetically different populations of *A. septentrionale* with low intrapopulational genetic variation in environmentally isolated localities may have been the result of such a strategy in Switzerland (Holderegger and Schneller 1994; Schneller and Holderegger 1996). The Estonian population can originate from a similar single-spore colonisation event – the nearest Finnish populations are only 40 km distant across the Gulf of Finland. The short history of the small, spatially isolated anthropogenic habitat in the location of Estonian population supports this hypothesis. In a photo from 1922, a newly completed stone wall with no soil or vegetation between the stones can be seen. This helped us to establish the approximate age of the Estonian population. However, the possibility that the population originated from spores in the local soil spore bank cannot be excluded.

The very high rate of intra-gametophytic selfing on isolated gametophytes of the Estonian population may actually reflect the level for isolated single spores in natural conditions. Sporophyte production of the species was significantly lower in bi-gametophytic systems (pairs of isolated gametophytes) in experimental conditions (Aragón and Pangua 2003), probably due to inter-gametophytic competition as proposed earlier for other fern species (Korpelainen 1996, 1997). Consequently, in natural populations, numerous gametophytes may be clumped together in a very small area of a ‘safe site’ (Harper 1977) and a considerable decrease in the production of sporophytes may be expected.

The results of common garden experiment demonstrated significant differences in fitness (estimated through plant morphology) among plants originated from Estonian and Finnish populations as Estonian plants were significantly smaller and, but a high mortality rate differed only from one Finnish population. The poor performance of Estonian plants in uniform common garden conditions is probably genetically related. Both Finnish habitats represent the typical habitat type of the species, i.e. cracks of acid, siliceous rock. The Estonian habitat, by contrast, is a human-constructed stone wall surrounding an earth mound. Plants grow between the stones in the outer side of the wall in acidic sandy soil. In some respect, the Finnish and Estonian habitats are similar; however, the Estonian habitat has some specific characteristics (e.g. soil origin). Considering the possibility of single-spore colonisation, and the relatively short history, the Estonian population may represent a specific randomly pre-adapted genotype, originating from founder effect and genetic drift. Another possibility is that a locally adapted old genotype might have re-emerged from the spore bank. The establishment of a single population, occupying a small specific habitat and incapable of spreading to very similar yet still somehow unsuitable habitats on the same island, may be the result of both scenarios. Further study is needed to analyse the populations’ genetic differences. Nonetheless, based on the biology of the species, and by taking into account the results of sporophyte formation and the common garden experiment, some basic recommendations can be proposed for the selection of suitable donor plant material for the introduction of *A. septentrionale*.

**Implications for conservation and introduction**

First, the high capacity of *A. septentrionale* for intra-gametophytic selfing determines its genetic characteristics, e.g. completely homozygous sporophytes, low genetic load (accumulation of deleterious, recessive alleles) and low inter-populational genetic diversity. Second, differences in mortality and morphological traits in the common garden experiment imply genetic differences among Estonian and Finnish populations. Further research is needed to ascertain the genetic diversity of the populations. Third, persistence of the self-sustaining (and viable) Estonian population indicates its high environmental suitability and the presence of a unique pre-adapted or locally adapted genotype, which needs conservation as an essential component of the species’ genetic diversity. More methodical and efficient protection of the local habitat would be the most effectual.

Fourth, to introduce *A. septentrionale* to a new, environmentally similar local habitat in Estonia, plant material (spores for propagation) should be collected from the local population. This would preserve the local genotype against unavoidable negative human impact. Fifth, the same donor plant material should also be used for reinforcement plantings (in order to reduce the risk of extinction of the population) in more or less stable environmental conditions of the local Estonian habitat. Sixth, in the case of severe environmental change and/or risk of extinction of the population, plant material (spores for propagation) for the reinforcement plantings should be collected from several different neighbouring habitats to maximise genetic diversity.

**Figure 4.** Mean with 95% confidence interval of number of leaves and plants height (length of the longest leaf) of *Asplenium septentrionale* sporophytes originating from the Estonian and two Finnish populations. Bars with the same letter are not significantly different (*P* < 0.05, Tukey HSD test).
diversity. Using source material with different characteristics for plantings increases the chance of finding a new suitable (pre-adapted) genotype and/or provides raw material for natural selection in a new site.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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