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Hilde Hens

POPULATION GENETICS AND POPULATION ECOLOGY IN MANAGEMENT OF ENDANGERED SPECIES

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POPULATION GENETICS AND POPULATION ECOLOGY IN MANAGEMENT OF ENDANGERED SPECIES

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Abstract

Knowledge of the determinants of the viability of populations is essential in order to undertake effective conservation and management of endangered species. In this study, long-term demographic data was combined with genetic data to study the viability of an endangered orchid species, *Epipactis atrorubens*. The genetic analyses revealed low levels of genetic variation and the presence of population genetic differentiation independent of the spatial scale. Low levels of seed-mediated gene flow, possibly linked to low seedling recruitment, is the likely cause of the low levels of gene flow. Indications of slow post-glacial colonisation rates were found, which together with the low gene flow predict a limited capacity of the species to shift its range to more suitable habitats after environmental change. Low genetic variation as a proxy for low evolutionary potential also suggests that the species has limited capacity to adapt to new environmental conditions. Furthermore, poor seedling recruitment lowers population viability in small populations, as highlighted by the low population growth rates. In addition, we found a strong effect of stochasticity that limits the viability of populations. Both the genetic and demographic analyses indicated low viability of the studied species and that seedling recruitment could be the main determinant for the viability.

Keywords: conservation, demography, genetic structure, genetic variation, population viability, post-glacial history

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Tiivistelmä

Luonnonsuojelun perusta on populaatioiden elinkykyyn vaikuttavien tekijöiden tuntemus. Tässä väitöskirjatyössä tutkittiin uhanalaisen orkidean, tummaneidonvaipan (*Epipactis atrorubens*), elinkykyyn vaikuttavia tekijöitä yhdistämällä pitkäaikaisseurannoilla kerätyt demografiset aineistot geneettisin menetelmin kerättyihin aineistoihin. Lajin populaatioiden geneettisen muuntelun määrän havaittiin olevan pieni ja populaatioiden todettiin olevan geneettisesti erilaistuneita maantieteellisestä skaalasta riippumatta. Geneettisen erilaistumisen syy voi olla alhainen geenivirta, joka on seurausta vähäisestä siemendispersaalista ja huonosta taimettumisesta. Populaatioiden evolutiivista historiaa tutkittaessa havaittiin merkkejä hitaasta jääkauden jälkeisestä kolonisaatiosta, mikä yhdessä alhaisen geenivirran kanssa ennustaa, että lajilla on huono kyky siirtyä sille sopivammille alueille, jos ympäristö muuttuu. Huonoa evolutiivista potentiaalia kuvastava vähäinen geneettinen muuntelu ennustaa, että lajilla on huono kyky sopeutua uusiin ympäristöoloihin. Tämän lisäksi huono taimettuminen laskee elinkykyä etenkin pienissä populaatioissa, mikä näkyy muun muassa pienten populaatioiden matalina kasvukertoimina. Stokastinen vaihtelu vaikutti elinkykyä alentavasti, mikä pitäisikin huomioida nykyistä paremmin elinkykyanalyyseissä. Sekä geneettiset että demografiset analyysit osoittivat taimettumisen mahdollisesti olevan määräävä tekijä tummaneidonvaipan populaatioiden elinkyvylle.

Asiasanat: demografia, geneettinen muuntelu, geneettinen rakenne, jääkauden jälkeinen historia, luonnonsuojelu, populaatioiden elinkyky

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Abbreviations

ABC	Approximate Bayesian computations
AMOVA	Analysis of molecular variance
A_R	Allelic richness
cpDNA	Chloroplast DNA
D_{est}	Jost's pairwise differentiation values
F	Fecundity
F_{IS}	Inbreeding coefficient
Fij	Pairwise kinship coefficient
F_{ST}/ Φ_{ST}	Fixation index
F_{STb}	Bi-parentally inherited differentiation coefficient
F_{STm}	Maternally inherited differentiation coefficient
G-W	Garza-Williamson Index
IBD	Isolation by distance
H_d	Haplotype diversity
H_E	Expected heterozygosity
HKY	Hasegawa-Kishino-Yano nucleotide substitution model
LGM	Last glacial maximum
M	Transition probability between stages
m_p/m_s	Ratio of pollen flow to seed gene flow
N	Total population size
N_0	Starting population vector
p	Recapture probability of the stages
PCA	Principle component analysis
PCR	Polymerase chain reaction
P_e	Extinction probability
TPM	Two-phase mutation model
π	Nucleotide diversity
θ_S	Watterson estimate of theta
λ_d	Deterministic growth rate
λ_s	Stochastic growth rate
Φ	Survival of the stages

Original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

I Hens H, Pakanen VM, Jäkäläniemi A, Tuomi J, Kvist L (2017) Low population viability in small endangered orchid populations: genetic variation, seedling recruitment and stochasticity. Biol Conserv 210: 174–183.

II Hens H, Jäkäläniemi A, Kvist L (2017) Restricted seed-mediated gene flow, fine-scale spatial genetic structure and low genetic diversity in a regionally endangered orchid. (Manuscript)

III Hens H, Jäkäläniemi A,Tali K, Efimov P, Kravchenko AV, Kvist L (2017) Genetic structure of a regionally endangered orchid, the dark red helleborine (*Epipactis atrorubens*) at the edge of its distribution. Genetica 145: 209–221.

Contents

1 Introduction

1.1 Extinction

The global biodiversity has been considerably reduced due to the direct and indirect human-induced extinctions. Globally, the conservation status of 79 800 species is assessed using the IUCN Red list criteria and more than 23 000 of them are threatened by extinction (IUCN 2015). According to the IUCN Red list criteria, 21% of the plant species are categorised as being threatened by extinction (Bachman *et al.* 2016). The main threats for plant species include human-induced habitat destruction, pollution and invasive species (Oostermeijer 2003). Habitat degradation and fragmentation have severe consequences for species and they have been shown to be a major threat to the viability of populations (Baillie *et al.* 2004). Habitat destruction can occur both directly, through for instance urbanisation, deforestation and conversion of habitats for agriculture, and indirectly through environmental changes.

Climate change can be a major threat to the persistence of species by changing the habitat conditions (Thomas *et al.* 2004). The suitable habitats of the species are expected to be changed and to decrease in numbers due to overgrowth (e.g. Dorrepaal *et al.* 2006) and environmental changes (Thomas *et al.* 2004). In addition to a global temperature increase, the predicted climate change includes alterations in precipitation patterns and an increase in the occurrence and strength of extreme events such as floods, droughts, high precipitation and extreme periods of heat (Easterling *et al.* 2000, Hartmann *et al.* 2013). The availability of suitable habitats may further decline indirectly due to climate change, as climate change can affect interactions with other species, such as mycorrhiza and pollinator communities (Micheneau *et al.* 2009, Fay *et al.* 2015). Arctic and boreal regions are reported to show the highest temperature increase (Stocker *et al.* 2013) and these high latitude regions such as the arctic tundra and parts of the boreal forest belt, are categorised among the regions most sensitive to climate warming (Root *et al.* 2003, Seddon *et al.* 2016). Northern species are adapted to harsh conditions, making them likely poor competitors for invasive species. In addition, they are often characterised by specific habitat conditions. Species living under very strict habitat conditions are especially prone to the threats of climate change (Baillie *et al.* 2004).

A major challenge for conservation is to preserve global biodiversity. In order to do so, we need to understand the dynamics underlying the threats which cause

species extinctions. Knowledge on the processes of population declines and eventually extinctions can help to assess extinction risks and enables the creation of appropriate conservation management strategies to prevent the extinctions of populations and species (Schemske *et al.* 1994, Bruna & Oli 2005).

1.2 Small population dynamics

The reduction of suitable habitats has significant consequences on the persistence of populations as they become smaller and fragmented, or even disappear. Small populations are prone to decreases in population size and eventually extinction due to demographic variation, i.e. stochasticity (Shaffer 1981, Lande 1988, Lienert 2004, Melbourne & Hastings 2008). Demographic variation can be caused by random fluctuations in extrinsic factors (e.g. climate conditions, resource availability), i.e. environmental stochasticity, random inter-individual differences, i.e. demographic stochasticity (Lande 1993), non-random differences among individuals (e.g. differences in quality at birth, difference in birth and death rates due to size or health variation), i.e. demographic heterogeneity (Kendall & Fox 2003), stochastically fluctuating sex ratios in the population (Engen *et al.* 2003, Saether *et al.* 2004) and sampling effects (Kendall & Fox 2002). Knowledge of the effect of demographic variation on the population viability is an important aspect of conservation biology (Lande 2002).

Allee effect and pollination limitation in plants are other ecological processes that can affect the viability of small populations. For example, pollination could be unsuccessful due to the lack of conspecifics or a low density, i.e. Allee effect (Groom 1998, Lamont *et al.* 1993, Courchamp *et al.* 1999). Lower reproductive success could also be affected by the amount or quality of pollen, i.e. pollen limitation. The occurrence of pollen limitation has been widely documented in many plant populations (Knight *et al.* 2005). Pollen limitation can occur due to the failure of small populations to attract pollinators, i.e. pollination limitation (Sih & Baltus 1987, Ågren 1996). Alternatively, pollen limitation has been suggested to be the result of reduced pollen quality, rather than the lack of pollen, due to a loss of compatible mates, reduced effectiveness of pollinators or altered pollinator behaviour (Aizen & Harder 2007, Wagenius & Lyon 2010). In addition to the effect of small population sizes, habitat fragmentation can also further affect the plant-pollinator interactions, as pollinators may move less among small and fragmented habitat patches (Goverde *et al.* 2002).

Understanding the importance of the different factors and processes that affect the persistence of populations is essential in order to avoid extinction. Population viability analyses can help to assess the extinction risk of populations, identify management plans and prioritise species for conservation and management. The components of population viability analyses include genetic processes, deterministic and stochastic population dynamics, and metapopulation dynamics. However, while population viability analyses have played a central role in conservation biology, the usage of population viability analyses as a tool to predict the extinction risk of species has been criticised, mainly due to its low confidence levels. For instance, the accuracy of the extinction probability estimates is often reduced by uncertainties due to factors such as the lack of knowledge of life-history parameters and factors important for the viability (Beissinger & Wesphal 1998, Mann & Plummer 1999, Coulson *et al.* 2001, Frankham *et al.* 2009, Zeigler *et al.* 2013). This knowledge is especially limited in small and endangered populations, which are in need of effective conservation measures the most. Furthermore, the exclusion of processes critical to population extinction and uncertainties might give rise to incorrect population viability estimates (Zeigler *et al.* 2013). Moreover, population viability analyses have been criticised due to a lack of empirical verification (Mann & Plummer 1999). However, population viability analyses have been the basis of successful conservation management (e.g. Yellowstone grizzly bear *(Ursus arctos horribilis*), Shaffer 1983, Boyce *et al.* 2001*)*. Lastly, it has been argued that modelled population demographic processes such as stochasticity, may not match the biological reality (Kendall & Fox 2002). Nevertheless, population viability analyses are important tools in conservation biology as they can be used to assess the importance of different parameters for the persistence of populations and species, and the outcomes of different management actions (Reed *et al.* 2002, Meek *et al.* 2015).

1.3 Genetic diversity

As genetic variation forms the evolutionary potential for species and populations to adapt to their environments, it is important for the long-term viability of a species (Ellstrand & Elam 1993, Young *et al.* 1996, Dierks *et al.* 2012). This becomes increasingly essential in a time of rapidly changing environments, which is presently caused by climate change and other human-induced changes such as pollution and the introduction of invasive species. Two types of genetic diversity can be distinguished: adaptive genetic diversity and neutral genetic diversity (Lowe

et al. 2004). Adaptive genetic diversity forms the raw material for natural selection and underlies both present adaptations and the evolutionary potential of populations (Frankham *et al.* 2009). The amount of genetic diversity in populations depends on the interplay of different processes. Mutations form the source of genetic diversity, where processes such as selection and random genetic drift cause the levels of genetic diversity to vary across time and space. Genetic variation is lost due to random genetic drift and the effect of drift increases with decreasing population size (Ellstrand & Elam 1993, Hedrick 2005). In addition, increased levels of inbreeding are expected in small populations as individuals will have a higher probability of reproducing with related individuals. Inbred individuals have a higher homozygosity than non-inbred ones, which can lead to the expression of harmful or even deleterious recessive alleles. The expression of such recessive alleles can decrease survival and reproduction and thus the fitness of individuals even in the short-term, i.e. inbreeding depression (Ellstrand & Elam 1993, Frankham 1995, Young *et al.* 1996, Reed and Frankham 2003, Leimu *et al.* 2006, Honnay & Jacquemyn 2007, Frankham 2010). Negative genetic consequences such as a loss of genetic diversity, inbreeding depression and genetic differentiation can be counteracted by gene flow. However, gene flow is often reduced due to habitat fragmentation. At present, population size reduction and fragmentation due to human-induced habitat degradation pose a major threat to both the long-term and short-term viability of species.

While there is extensive theoretical background on the negative consequences of genetic processes on the viability of populations, the impact of these processes on the extinction of species has been questioned (Lande 1988, Gilligan *et al.* 1997, Schmidt & Jensen 2000). It has been suggested that populations will only suffer from genetic processes after they have experienced critically declines in size due to ecological processes (Lande 1988, Schmidt & Jensen 2000). Schmidt & Jensen (2000) showed that populations are only considered to be in an early phase of genetic erosion even after experiencing severe population declines, as indicated by the high levels of genetic variability in small populations. No examples of extinction due to genetic factors has been documented as of yet (Begon *et al.* 2006).

However, it has been shown that endangered species do have lower genetic variation as compared to non-threatened related species (Spielman *et al.* 2004). In addition, studies have pointed out reduced genetic variation (Young et al 1999, Buza *et al.* 2000, Gaudeul *et al.* 2000, Luijten *et al.* 2000, Leimu *et al.* 2006, Honnay & Jacquemyn 2007, Aguilar *et al.* 2008) and increased inbreeding (Oostermeijer *et al.* 1994, Buza *et al.* 2000) in small populations. Also, increased

levels of inbreeding have been shown to be related to a reduction in fitness in small populations (Keller & Waller 2002, Reed & Frankham 2003, Blomqvist *et al.* 2010). Furthermore, loss of genetic diversity has been shown to have a negative impact on reproduction (e.g. number of seeds/capsules, fruit set, seed mass) and survival (e.g. resistence against pathogens, plant size) (Paschke *et al.* 2002, Hensen & Oberprieler 2005, Lauterbach *et al.* 2012, He 2013). Consequently, preserving genetic variation and gene flow is important for the viability of populations and knowledge of the levels of genetic diversity is needed to assess the ability of populations to cope with environmental changes. However, selectively neutral genetic diversity does not directly affect the individuals fitness nor can it be used to assess the adaptive genetic variation directly. On the other hand, studying neutral genetic diversity can give insights on evolutionary processes.

1.4 Genetic structure

The distribution of the genetic diversity among populations, i.e. spatial genetic structure, is determined by genetic processes such as genetic drift, gene flow and selection (Frankham 2005). Populations will differentiate due to the processes of genetic drift and directional selection towards separate phenotypes if there is limited gene flow. The dispersal rates and thus gene flow among populations are often reduced or limited due to geographical barriers for migration and habitat fragmentation. Genetic studies can provide insights into the processes such as population structure and dispersal events among populations, which is valuable information for conservation and management plans.

In addition to the current dynamics, historical change in the distribution of species has contributed to the present geographic and population genetic patterns of species. Boreal and temperate species in North-West Europe have experienced recurring distributional shifts caused by repeated glaciations during the Pleistocene (Hewitt 1999, Hewitt 2000, Hewitt 2004). During glaciations, species shifted their ranges to ice-free refugia and expanded their ranges again by following the retreat of the ice sheets during interglacials (Hewitt 1999, Hewitt 2000, Hewitt 2004). Populations in refugia often experienced bottlenecks and were isolated from populations in other refugia due to geographical barriers, thereby leading to a loss of genetic variation and population differentiation (Hewitt 1999, Hewitt 2004, Arenas *et al.* 2012, Pellissier *et al.* 2016). Genetic variation can be further lost during interglacials, as the leading-edge contained lower levels of genetic diversity during range expansions due to recurrent founder events, i.e. founder effect (Hewitt

1999, Hewitt 2004, Arenas *et al.* 2012). These historical colonisation events of species have therefore left clear genetic signatures on the current genetic differentiation and diversity patterns. By studying the patterns of spatial genetic differentiation, knowledge on the colonisation history of species can be acquired. Knowledge on the effects of the past environmental changes can also be utilised to predict consequences of environmental changes also in the future (Hampe & Petit 2005, McLachlan *et al.* 2005, Svenning & Skov 2007, Bhagwat & Willis 2008, Provan & Bennett 2008, Hampe & Jump 2011).

According to phylogeographic studies on both plant and animal taxa, the European biota is assumed to have been restricted to three main southern refugia during the last glacial maximum (LGM), namely the Mediterranean peninsulas of Iberia, Apennine and the Balkans (Taberlet *et al.* 1998). However, the presence of additional northern refugia during the LGM has been proposed by Hultén (1937). Eastern Eurasia has been proposed as a refugium for arctic and boreal species, as large areas of Eastern Eurasia remained ice-free during the LGM (Svendsen *et al.* 1999) and support for such refugia located in the East has been found based on phylogeographic studies and fossil data on both animal (Flagstad & Røed 2003, Zigouris *et al.* 2013) and plant species (Tremblay & Schoen 1999, Abbott *et al.* 2000, Stenström *et al.* 2001, Brubaker *et al.* 2005). In addition, species may have persisted in favourable micro-refugia within the ice sheets (Holderegger & Thiel-Egenter 2009, Rull 2009). Support for such micro-refugia in Scandinavia during the LGM has been found for several tree species (Kullman 2008, Westergaard *et al.* 2011, Parducci *et al.* 2012). Northern Norway and southern Norway have been identified as suitable places for northern micro-refugia, as parts of the Norwegian west-coast remained ice-free throughout most of the LGM. However, theories on the occurrence of these refugia have been criticised by other studies (Vorren *et al.* 2013). Additionally, it has been proposed that ice sheets covered with debris may have been suitable for plant growth and may have functioned as refugia during the LGM or allowed for early colonisation by plant species after the LGM (Fickert *et al.* 2007). Consequently, such debris-covered ice sheets could have functioned as colonisation substrates for plant species for the other parts of Scandinavia.

1.5 Orchids

The family Orchidaceae is, with over 25 000 described species, the largest flowering plant family (Givnish *et al.* 2015, Govaerts *et al.* 2016). Although the abundance and distribution of many orchid species are skewed towards the tropics,

orchid species can be found on all continents except Antarctica. Orchid species typically rely on the interactions with specific pollinators and mycorrhizae for their reproduction and survival.

Orchids produce very small seeds that lack sufficient nutrient reserves to produce autotrophic seedlings (Bernard 1889, Bernard 1990, Arditti & Ghani 2000). Seed germination therefore requires the presence of mycorrhizal fungi (Rasmussen 1995). In addition, orchids depend on the association with mycorrhizal fungi for its carbon supply during the protocorm stage (Rasmussen 1995). Furthermore, some orchid species also rely on mycorrhizal fungi in the later stages of their life-cycles (Leake 1994). Some orchid species have been shown to be highly specific with regards to the fungal families, genera or even specific species on which they are reliant (Taylor *et al.* 2002) and the distribution of populations has been shown to be limited by the distribution and abundance of their mycorrhizal fungi (McCormick *et al.* 2012). However, a wide diversity of fungal associates has been found in orchids as well (e.g. Stark *et al.* 2009). In addition, orchids rely on pollinator species for their sexual reproduction (Micheneau *et al.* 2009). Orchids are pollinated by a wide range of pollinator species including birds, moths, butterflies, flies, bees and wasps (Tremblay *et al.* 2005). Some orchid species are also highly specific with regards to their pollinator species (Tremblay 1992, Schiestl & Schlüter 2009), which sometimes show remarkable co-adaptations (Michaneau *et al.* 2009).

The specificity of orchid species with regards to their biotic associations makes them vulnerable to the negative impact of habitat degradation and environmental changes (Micheneau *et al.* 2009, Fay *et al.* 2015). Proportionally, the orchid family contains the highest number of endangered species out of all plant families (Swarts & Dixon 2009a). In Europe studies have reported strong range contractions for a large proportion of orchid species in Belgium, the Netherlands (Jacquemyn *et al.* 2005), Estonia and the United Kingdom (Kull & Hutchings 2006). Even cases of extinctions have been documented (Jacquemyn *et al.* 2005). Due to specific habitat conditions, orchids are recommended as bio-indicators for the health of habitats, as changes in the normal habitat conditions is expected to influence their population dynamics and their population viability.

Successful conservation of orchid species requires knowledge on the consequences of habitat reductions and fragmentations, and environmental changes (Swarts & Dixon 2009b). Demographic studies and conservation of orchids are complicated by the complex life history characteristics of orchids, such as long generation lengths, vegetative dormancy, presence of seed banks and complex

interactions with mycorrhizal fungi and pollinators. Due to the occurrence of undetectable underground vegetative dormant stages, dormant individuals cannot be distinguished from dead individuals based solely on above ground observations, which leads to underestimates in population size (Shefferson *et al.* 2001). Consequently, demographic studies in orchids require long-term monitoring (Lesica & Steele 1994). Furthermore, biotic interactions can be altered in many different ways by environmental changes and habitat fragmentation, making the conservation of orchid species even more challenging (Sletvold *et al.* 2013, Fay *et al.* 2015).

1.6 Aims

While genetic and ecological processes are both important for the viability of species, the combination of both processes has rarely been applied to conservation studies. My main aim in this thesis was to assess the viability of populations of an endangered species using genetic and demographic data and to create guidelines for conservation purposes. Firstly, I studied the viability of populations using long-term demographic data. Using several model approaches, I estimated extinction probabilities and pinpointed the life history characteristics important for the viability. Secondly, I studied the genetic diversity and population genetic differentiation patterns both on local and large spatial scales to get insights on the genetic viability of the species, and knowledge on the dispersal patterns and the amount of gene flow. I also assessed the importance of the genetic and demographic processes for the population viability. Additionally, I looked for indications of historical processes that underlie the present geographical distribution and genetic structure.

2 Materials and methods

2.1 Study species

Epipactis atrorubens (Hoffm. Ex Bernh.) Besser (Dark-red Helleborine) is a perennial orchid species with a wide distribution, extending from Europe to the Ural Mountains in Asia and western Siberia (Fig. 1). In Europe, the main distribution is located in the centre and the east, but populations can also be found further up north all the way to the northernmost parts of Norway, Southern Sweden and Finland.

According to the IUCN red list criteria, *E. atrorubens* is currently not categorised as endangered in Europe (Least concern; Bilz *et al.* 2011). However in some countries or regions the species has been categorized as critically endangered (Belgium, the Netherlands), endangered (Greece), vulnerable (Belarus, Czech Republic, Denmark, Finland, Lithuania, Luxembourg, Ukraine) or near threatened (Hungary) (Moser *et al.* 2002, Wind & Pihl 2004, Cheffings & Farrell 2005, Colling 2005, Saintenoy-Simon *et al.* 2006, Van Landuyt *et al.* 2006, Zarzycki & Mirek 2006, Zarzycki & Mirek 2006, Király 2007, Rasomavièius 2007, Moreno 2008, Tartes *et al.* 2008, Didukh 2009, Hohla *et al.* 2009, Petrova & Vladimirov 2009, Phitos *et al.* 2009, IUCN France *et al.* 2010, Kålås *et al.* 2010, Rassi *et al.* 2010, Grulich 2012, Jelić *et al.* 2012, Rossi *et al.* 2013, HBC 2014, Sparrius *et al.* 2014, Eliáš *et al.* 2015, Westling 2015).

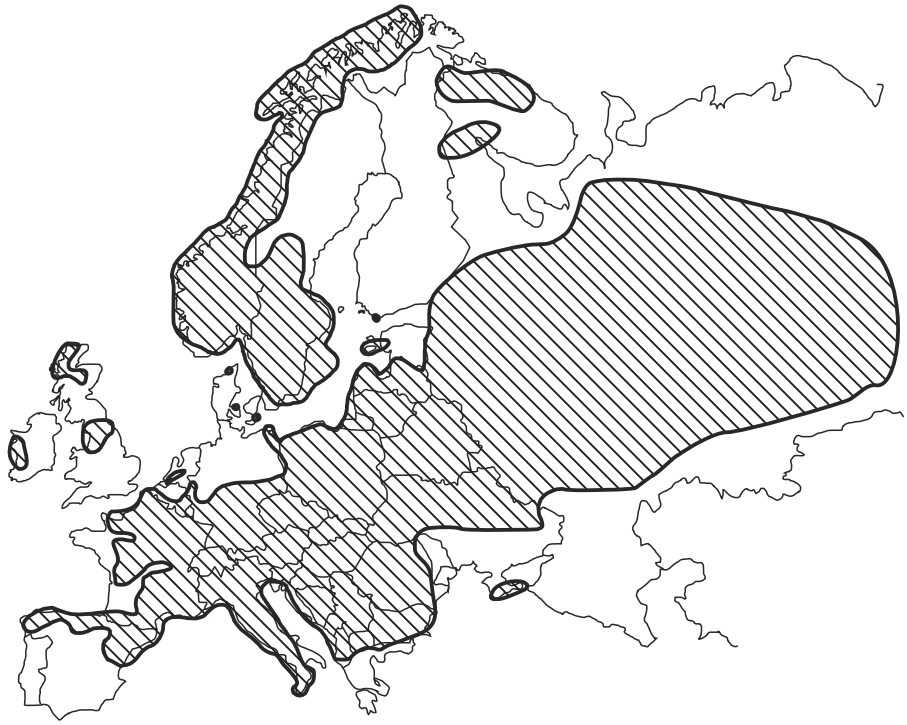

Fig. 1. Distribution map of *Epipactis atrorubens* in Europe. The map was assembled from local distribution maps if available (Kuusk *et al.* 2003, Efimov 2004, Rasomavièius 2007, BSBI & BRC 2008, Didukh 2009, Phitos *et al.* 2009, Anthos 2010, CRSF & ZDSF 2012, NetPhyt 2013, Nimis *et al.* 2013, Lampinen & Lahti 2015, NDFF & FLORON 2015, Artdatabanken 2016, BfN 2016, ČNFD 2016, DGRNE 2016, Julve 2016, Natuurpunt 2016, NBIC & GBIF-Norway 2016, Snowarski 2016) or from European distribution maps (Euro+Med 2011, Rankou 2011) (III).

The species is characterised by its specific habitat conditions as populations are only found on slopes with limestone or dolomite. *E. atrorubens* has a predominantly allogamous reproduction, in which flowers are pollinated by wasps and bees (Brzosko *et al.* 2006, Tałałaj & Brzosko 2008, Jakubska-Busse & Kadej 2011). The seeds are dispersed by wind and can only germinate when appropriate mycorrhizae are available. After germination, individuals develop in an underground protocorm stage before seedling recruitment. After seedling recruitment, an individual grows in a vegetative stage for several years (average 7.4 years ± 2.29; I). Individuals overwinter as an underground rhizome and could potentially go into dormancy for one or more year(s) (Jäkäläniemi *et al.* 2011). On

average, 16.3% of the total population remains dormant (I). The length that individuals remain dormant varies greatly from one to thirteen years (average 1.34 years; I).

2.2 Sampling

Samples for the genetic analyses were collected from seven different regions, in Estonia (Hiiumaa N=60), Russia (Karelia N=30, Vologda N=17, Psvok N=17) and Finland (Oulanka N=761, Juankoski N=30, Hanko N=30) (III) (Fig. 2). In the northernmost region, or more specifically the Kuusamo region, a more thorough sampling procedure was used, in order to study detailed, fine-scale spatial genetic patterns. Samples in this region were collected from forty different populations of various sizes (II). One leaf was collected from each individual and was frozen at -20ºC or dried using silica gel. As individuals can ramify above the ground surface from the root neck, one genet can have several shoots (1–28 shoots; I) belonging to the same rhizome. Therefore special attention was paid to ensure that the samples represented genetically distinct individuals. Samples were taken from individuals that were clearly separated and only one leaf was collected from a group of shoots belonging to one individual.

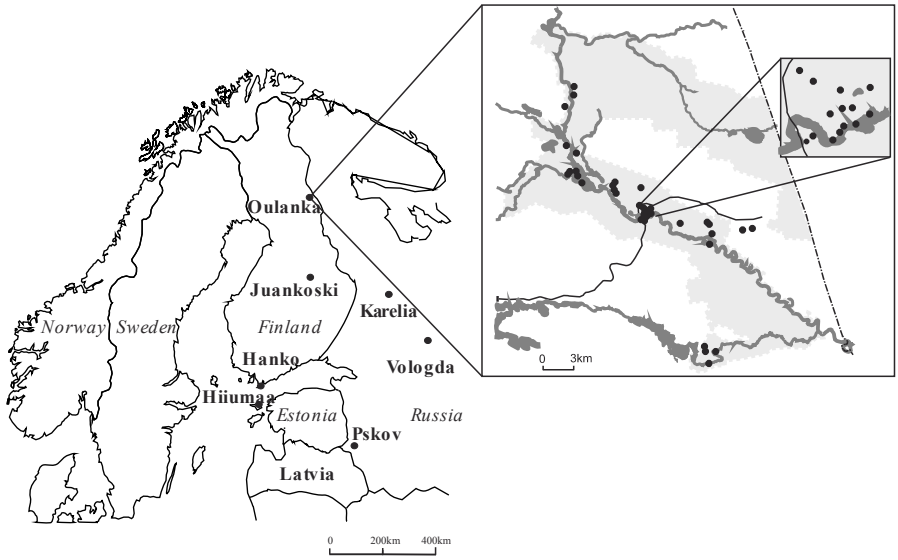

Fig. 2. Map of the sampled regions; Oulanka, Juankoski, Hanko in Finland, Karelia, Vologda and Psvok in Russia and Hiiumaa in Estonia.

2.3 Demographic monitoring

To assess the demographic dynamics of populations, eleven populations were monitored in Oulanka (I). Three populations were part of a long-term monitoring project and have been monitored since 2000 or 2002 (for 16 and 14 years, respectively). As these populations were all large populations (>100 individuals), additional small populations were included to enable the effect of population size to be studied. Monitoring of these additional populations began in 2010 or 2013 (i.e. monitoring duration of 6 and 3 years, respectively) (Table 1). The populations varied in size from 11 to 313 monitored individuals. In the large populations (N>100 individuals) only individuals within an area of 10 m x 10 m were monitored whereas in the small populations (N<100 individuals) all individuals within the population were monitored. In the first year of the monitoring all individuals within the population or monitoring plot were tagged using plastic tags with ID numbers and censused in the following years, during the flowering time (early August). All new individuals were tagged each year and all individuals were characterised by the number of shoots, flowers, capsules and the height of the plant.

2.4 Demographic methods

In plant population viability analyses, matrix models are the most commonly used method. In many analyses, only short term data is used while many factors, such as genetic variation, stochasticity and many life-history characteristics like dormancy, seed banks and clonal reproduction are ignored (Zeigler *et al.* 2013). In this study, the demographic dynamics in the populations were studied using matrix modelling and mark-recapture methods.

2.4.1 Life history cycle

For the demographic analyses, individuals were classified into eight life stages (Fig. 3) (I). A seedling (s) was defined as a newly-emerged individual shorter than 3cm in height, with less than three leaves. A vegetative individual (v) was defined as an individual that had not been observed as a flowering individual in a previous year and has a maximum of one shoot. A mature vegetative (mv) individual was classified as an individual with more than one vegetative shoot or a vegetative individual that had been observed as a flowering individual in a previous year. A fertile individual (f) was defined as an individual with at least one flowering shoot. A dormant individual was defined as an individual that does not emerge above ground, but re-emerges in a later year. As it has been shown that the life stage before the dormant stage does affect the performance of a dormant individual after its dormancy (Jäkäläniemi *et al.* 2011), four dormant stages were defined, based on the stage before dormancy. They are dormant seedling (ds), dormant vegetative (dv), dormant mature vegetative (dmv) and dormant fertile (df).

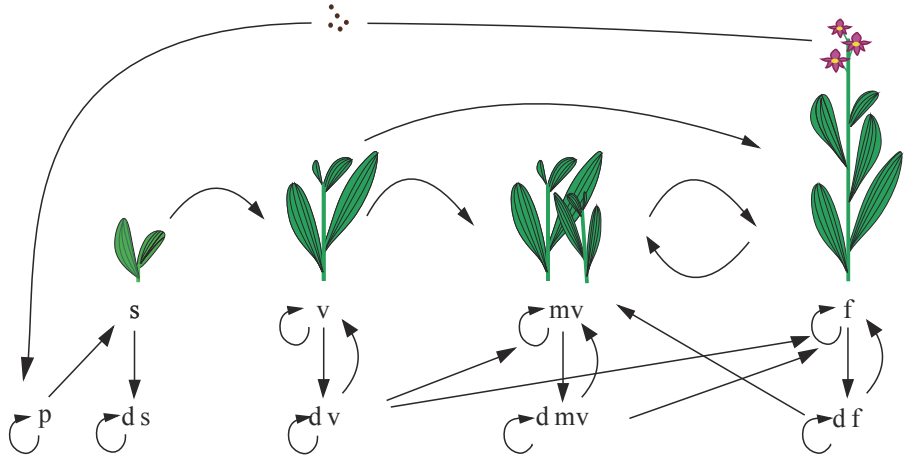

Fig. 3. Stage-based life cycle graph for *E. atrorubens*. The life stages include protocorm (p), seedling (s), vegetative (v), mature vegetative (mv), fertile (f), dormant seedling (ds), dormant vegetative (dv), dormant mature vegetative (dmv) and dormant fertile (df). Arrows represent possible transitions.

In this study, an individual was classified as dead when it did not emerge after three consecutive years or more, as carried out by Shefferson (2009). In the monitored populations, dormant stages of thirteen successive years have been observed and a bias towards dead individuals in the last years of the monitoring can occur. However, as the average dormant stage lasted less than three years (average= 1.34 years), the error due to this was assumed to be small. Individuals that did not emerge during the last three monitoring years cannot be classified as dormant or dead solely based on above-ground observations. Therefore, the last monitoring years of these individuals were excluded from the data. Death rates in the populations with a short monitored period could not be defined and therefore the average mortality rate of populations with a long-monitored period was used.

After seed germination individuals develop in a protocorm stage before emerging above ground as seedlings but the time between seed production and seedling emergence is unknown. In order to test for the length of the time lag between seed formation and seedling recruitment, a generalized linear model was used, comparing the likelihood of different models with different time lag lengths. However, no clear inferences about the best time lag length could be made, as the AICc values decreased with the number of years. Therefore, the fecundity of year t was calculated as the number of seedlings in year t divided by the average number of fertile individuals in the years t-x. The transition probability from protocorm to

small vegetative was set to 1. No density dependence was included in the models, due to the lack of a clear correlation between the number of seedlings and the number of above-ground plants (R^2 = -0.073–0.0003, p = 0.160–0.999).

2.4.2 Deterministic and stochastic matrix models

The demographic census data was used to construct annual transition matrices for each population separately (I). In total, fifteen, thirteen, five or two annual transition matrices were constructed per population for the populations monitored for sixteen, fourteen, six and three years, respectively. A mean population matrix was constructed using the annual transition matrices. For each population, the finite rate of population growth (λ_d = dominant eigenvalue of the transition matrix; hereafter the deterministic growth rate) was calculated using the PopTools package v3.2 (Hood 2010) in Excel (Caswell 2001).

Stochastic matrix models were used to assess the population viability further using the annual transition matrices (I). Due to the short monitoring times of the small populations and Mataraniemi, data was lacking in several transitions in the individual transition matrices. Therefore, these populations were excluded from the stochastic analyses and stochastic matrix models were only applied to the three large populations monitored since 2000 (Ampumavaara and Kiutaköngäs N) and 2002 (Patoköngäs). The stochastic growth rate (λ_S) and probability of extinction (P_e) (Morris & Doak 2002) were calculated using the POPBIO v3.2 package (Stubben & Milligan 2007) in R. In this analysis, the annual matrices were selected at random with replacement (each matrix had an equal probability of selection). Simulations were performed for ten runs using 5000 simulations and 500 years into the future. A starting population vector (N_0) was constructed based on the size distribution of the population in the year 2012, as this was the latest year when all stages could be defined (including the dormant stages). To do so, all individuals inside and outside the 10 m x 10 m plots were counted during 2012 and the stage class distribution from inside the plot was used to extrapolate the total number of individuals for each stage class for the starting population vector (N_0). Populations containing less than 25 individuals were defined as functionally extinct (quasi-extinction threshold). A population with an extinction probability smaller than 5% over the next 100 years was considered to be a viable population (Menges 1990).

The effect of population size on the population viability was studied by assessing the extinction probability (P_e), by combining the annual population transition matrices of the three large populations to estimate the extinction

probability as described above, using an initial population size of 50, 100, 200 and 500 with the average stage distribution of the large populations (I).

The Pearson product-moment correlation test was conducted to test for a correlation between the total population size and the fruit set (percentage of flowers that set fruit in the population), population growth rate (λ_d) and fecundity. Fecundity and deterministic growth rate (λ_d) were compared between small and large populations using a one-way ANOVA.

The influence of genetic variation on the population viability was assessed by testing for a correlation between the genetic variation estimates (A_R, F_{IS}, H_E), the population growth rate (λ_d) and the total population size using the Pearson product-moment correlation (I). In addition, the genetic variation estimates (A_R, F_{IS}, H_E) of the small populations were compared to those of the large populations using a one-way ANOVA (I).

2.4.3 Capture-recapture analyses

The individual survival level was further analysed using capture-recapture analysis, in which demographic data was combined with genetic data in the program MARK v8.0 (White & Burnhamb 1999) (I). Two models were compared to analyse the survival of individuals and to examine whether survival is affected by individual heterozygosity. The two models used were a global model with only the stage-dependent model parameters (Φ(stage) p(stage) M(stage)) and a model, in which the survival of different stages was constrained by individual heterozygosity values (Φ = survival of the stage, p = recapture probability of the stages and M = transition probability between stages). The models were compared using AICc-values, where the difference of two AICc units was considered in order to infer real support for a variable (Burnham & Anderson 2003). The goodness of fit was examined using the median ĉ-approach, which suggested no overdispersion (ĉ = 0.96, 95% CI 0.87–1.04). In this analysis, data from three populations (Kiutaköngäs N, Ampumavaara and Patoköngäs) was pooled and only individuals that were genotyped using microsatellites were included. In the capture-recapture models, the recapture probability was used to estimate the survival of individuals (Lebreton *et al.* 1992). The recapture probability (p) was used to estimate the probability of being dormant as 1-p (Shefferson *et al.* 2001). Multi-state models were used as different stages can have different survival rates. The global model included three populations and four stages, Φ(pop+stage) p(pop+stage) M(pop+stage). The following probabilities were fixed at zero: recapture probability of seedlings, transitions from more

advanced stages to seedlings and from mature stages to vegetative stages. Time dependence was not included in the model because the number of parameters would have become too large with respect to the amount of data. Instead, the goodness of fit was examined using the median ĉ-approach, which suggested no overdispersion (ĉ = 0.97, 95% CI 0.79–1.14). Individual heterozygosity was included as a co-variant in the second model.

2.5 Genetic analyses

2.5.1 Molecular methods

DNA was extracted from the leaf samples using a modified 2x cetyltrimethylammonium bromide protocol (Doyle 1987) (I, II, III). Both chloroplast and nuclear molecular markers were used in this study to assess the genetic diversity and structure (II, III). Chloroplast markers are haploid and mostly maternally inherited in angiosperms, whereas nuclear markers are diploid and bi-parentally inherited. Chloroplast DNA is often characterised by low mutation rates.

To assess the chloroplast genetic variation and differentiation, five chloroplast loci were tested for amplification. However, due to the low amplification success two loci (trnS-trnG intergenic spacer and trnQ-rps16 intergenic spacer; Shaw *et al.* 2007) were excluded and three of them (trnL intron; Taberlet *et al.* 1991, trnK^{uuu} intron; Johnson & Soltis 1994, intergenic spacer of the rbcL-accD region; Tranchida-Lombardo *et al.* 2011) remained for sequencing and further analyses (II, III). The use of the trnL loci was limited to study of the fine-scale spatial genetic patterns (II).

In order to study the nuclear genetic variation, fifteen nuclear microsatellites were tested for amplification (I, II, III). Due to the low amplification success, low repeatability or the absence of genetic variation, nine nuclear microsatellites were excluded for further amplification (DO-06, DO-07; Gu *et al.* 2007, SW1-144, SW1-60, SW2-152; Tranchida-Lombardo et al 2008, EPP12, EPP86; Pinheiro *et al.* 2008, Lspe-1, Lspe-3; Cortés-palomec *et al.* 2008) and six nuclear microsatellites were thus used for further analyses (SW1-76, SW1-78, SW1-120, SW2-185, SW2-152; Tranchida-Lombardo *et al.* 2008, Ccal25; Minasiewicz & Znaniecka 2014). Detailed protocols for PCR amplifications can be found in the original articles (I, II, III).

2.5.2 Genetic variation

The genetic variation in the chloroplast sequences was assessed by calculating estimates for haplotype (h_d) and nucleotide diversities (π) (Nei 1987) with ARLEQUIN v. 3.5 (Excoffier & Lischer 2010) (II, III). Watterson's estimators of theta (Θ_S; Tajima 1989) were calculated manually to account for the length difference of the sequence (II, III).

Similarly, the nuclear genetic variation was estimated by calculating the expected heterozygosity (H_E) using ARLEQUIN v3.5 (Excoffier and Lischer 2010) and the allelic richness (A_R corrected for sample size using the rarefaction method) with HP-RARE v.1 (Kalinowski 2005) (I, II, III). The allelic richness was estimated for populations with at least nine sampled individuals.

2.5.3 Genetic differentiation

The genetic differentiation between subpopulations was tested for chloroplast and nuclear markers separately using different methods. The tests were conducted on a fine and a large spatial scale separately. Firstly, F-statistics (Wright 1950) were used to test for the occurrence of genetic structuring. Pairwise F_{ST}- and Φ_{ST}-values were calculated between the predefined subpopulations as measurements of population differentiation (II, III). An analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) was conducted to test for the overall differentiation among the subpopulations (II, III). Both the pairwise F_{ST}-values and AMOVAs were conducted using ARLEQUIN v3.5 (Excoffier & Lischer 2010). In addition to the pairwise F_{ST}-values, Jost's pairwise differentiation values (D_{est}; Jost 2008) were calculated using SMOGD v1.2.5 (Crawford 2010), as they have been shown to be independent of the within-subpopulation gene diversity, and are unbiased with regards to the sample size (II, III). F-statistics have been shown to give biased results when the genetic diversity is high. However they perform better when the genetic diversity is low. As a significant correlation (R^2=0.464, $p<0.001$ and R^2=0.495, p=0.023, tested using a Pearson's product-moment correlation in R) was found between the F_{ST}-values and the D_{est}-values, only F-statistics were used hereafter (II, III).

In order to test for the presence of isolation by distance (IBD) on an individual level, by calculating the correlation between the mean pairwise kinship coefficient (Fij; Loiselle *et al.* 1995) and the natural logarithm of the geographical distances between the individuals (II, III). This test was conducted with the program

SPeGeDi v 1.5 (Hardy & Vekemans 2002) using either nine (large spatial scale) or ten (small spatial scale) distance classes. Standard errors were estimated using the jackknife method over loci and the significance was tested using a one-tailed permutation test (10 000 permutations).

The nuclear genetic structure was further investigated using the program STRUCTURE v2.3 (Pritchard *et al.* 2000, Falush *et al.* 2003) (II, III). In this test, the best number of clusters is assessed based on the occurrence of a Hardy-Weinberg equilibrium and linkage equilibrium within clusters. This test was again conducted on both a fine and a large spatial scale. On both scales, one to ten clusters were tested for their likelihood. No *a priori* information of the sub-populations was used. An admixture model and correlated allele frequencies between populations were assumed. In total, ten replicates of each number of clusters were performed, using a burn-in period of 100 000 and 500 000 MCMC repeats after the burn-in. The ΔK value which refers to the second order rate of chance of likelihoods between consecutive values of Ks, was calculated based on the Evanno method (Evanno *et al.* 2005) using STRUCTURE Harvester v 0.6.94 (Earl & vonHoldt 2012) to infer the most likely number of clusters. The ΔK values have been shown to perform better than the likelihood (LnP(K)) values in predicting the real number of clusters. The likelihood of individuals belonging to one of the clusters was assessed by calculating individual assignment probabilities to the obtained clusters. The assignment probabilities were plotted on geographical maps. The membership coefficients of the repeated runs in STRUCTURE were aligned using CLUMPP v1.1.2 (Jakobsson & Rosenberg 2007) and the bar plots representing the individual assignment probabilities of study populations were constructed using Distruct v. 1.1 (Rosenberg 2004).

2.5.4 Gene flow

Estimates of genetic differentiation can give insights on the levels of gene flow between populations. Low levels of genetic differentiation are expected when there is an extensive amount of gene flow among populations, as gene flow counteracts genetic differentiation due to genetic drift and/or selection. In addition, assignment analyses can be used to identify first generation migrants between the populations. A Bayesian assignment analysis was conducted using Geneclass 2.0 (Piry *et al.* 2004), through the method described by Rannala & Mountain (1997) (II). The resampling method of Paetkau *et al.* (2004) was used to estimate the probability using 10 000 simulations. The alpha level (type 1 error) was set to 0.01 and the

assignment threshold to 0.05. The number of individuals which were assigned as first generation migrants in this analysis cannot always be interpreted as exact numbers of migrants, because unsampled source populations can lead to a failure in categorising individuals as migrants or in the assignments of individuals to wrong source populations. Additionally, many pairwise comparisons based on a large sample size could lead to incorrect assignment of individuals as migrants just by chance (Rannala & Mountain 1997). However, the assignment results can give indications on the amount of gene flow among the sub-populations.

Gene flow in orchids occurs through seeds and pollen. While both nuclear and chloroplast DNA are dispersed by seeds, only nuclear DNA is dispersed by pollen. By comparing the differentiation estimates based on maternally inherited chloroplast and bi-parentally inherited nuclear markers, the relative contribution of gene flow due to pollen and seeds can be estimated (Ennos 1994). In order to do so, the ratio of pollen flow to seed gene flow (m_p/m_s) was calculated according to Ennos (1994) (II). The method of Hamilton & Miller (2002) was used to test for a significant difference between pollen and seed flow (II). The expected differentiation of the maternally inherited marker (F_{STm}) can be estimated based on the bi-parentally inherited differentiation (F_{STb}) as $F_{STm} = (a_1F_{STb})/(a_2+(a_1-a_2)F_{STb}$, where a_1 and a_2 are constants accounting for the difference in the organelle and nuclear fixation indices. The appropriate values of a_1 and a_2 depend on the mating system (e.g. outcrossing rate) and the used organelle DNA which is either maternally inherited or bi-parentally inherited. For outcrossing hermaphrodite plants, a_1 and a_2 are equal to 6 and 2 respectively. The null hypothesis of equal pollen and seed flow was tested using the 95% confidence intervals of the observed and expected F_{STm} values, generated by bootstrapping using the script of Hamilton & Miller (2002).

2.5.5 Past population processes

Past population size changes can leave their mark on the genetic pattern of species and there are multiple ways to find indications of the past population size changes. A recent reduction in the population size will cause an excess in heterozygosity, as rare alleles, that contribute little to the heterozygosity, will be lost first (Cornuet & Luikart 1996). The significance of a heterozygosity excess was tested using the Wilcoxon sign rank test (III). In addition, a mode-shift test was conducted to find deficiencies in low frequency allele classes caused by a bottleneck (III). The Wilcoxon sign rank test and the mode-shift test were conducted in

bottleneck1.2.002 (Piry et al, 1999) using the two-phase mutation model (TPM) with 70% single step mutations. The Garza-Williamson Index (G-W; Garza & Williamson 2001) was calculated with Arlequin v. 3.5 (Excoffier & Lischer 2010) in order to find indications of gaps in the allele size distribution, as such gaps could indicate a recent bottleneck since the number of alleles will be lost faster than the allelic range which narrows in decreasing populations (III). Due to the low genetic variation in the studied chloroplast loci, it is unlikely that these tests would give reliable predictions on the past population size changes and therefore only the nuclear microsatellite data was tested.

Coalescent-based approximate Bayesian computations (ABC; Beaumont *et al.* 2002) using DIYABC v2.1.0 (Cornuet *et al.* 2014) were applied to compare different scenarios of the colonisation origin of the studied populations. Three hypotheses were tested (1) refugia located in southern Europe with a recolonisation following the retreat of the ice sheets from south to north (Scenario 1), (2) a colonisation origin from refugia located east from the sampled regions with a recolonisation following the retreat of the ice sheets from east to west (Scenario 2), (3) two independent refugia located in the South and the East, resulting in a secondary contact zone, where the two colonisation routes meet (Scenario 3) (Fig. 4). In these analyses, both nuclear microsatellite and chloroplast sequence data were used. The mutation rate was set as uniform with a range of 1.1^{-9}–2.9^{-9} and 10^{-2}–10^{-5} substitutions per synonymous site per year for the chloroplast loci and nuclear loci respectively. This rate was chosen based on the mutation rates of chloroplast DNA and nuclear microsatellites among different plant species (Muse 2000, Udupa & Baum 2001, Thuillet *et al.* 2002, Vigouroux *et al.* 2002, Marriage *et al.* 2009). The Hasegawa-Kishino-Yano (HKY, Hasegawa *et al.* 1985) nucleotide substitution model was used for the chloroplast loci. Secondary contact zones were simulated by an admixture between two colonisation routes. The rate of admixture was set as a uniform distribution (0.001–0.999). Since the exact population sizes were unknown, the prior distribution of effective population sizes was set as uniform with a large range (1 000–10 000). The prior distributions of the divergence times were chosen based on reconstructions of the deglaciation after the LGM and the evolution of the Baltic Sea (Svendsen *et al.* 1999, Tikkanen & Oksanen 2002, Kallio 2006). Given that divergence patterns could have been formed before the actual recolonisation, which can be a slow process, a large range was chosen. The divergence times are expressed in generations and 25 years was set as the generation time for *E. atrorubens*. Since no data was available from additional eastern, southern or northern Norwegian populations, an unsampled refugial

population was included in scenario 3 (Fig. 4). Hanko was excluded from these analyses, due the known fact that its colonisation has been recently. A principal component analysis (PCA) in space of the summary statistics, based on simulating 600 000 datasets, was used to evaluate how the observed data is surrounded by the simulated datasets. Necessary adjustments to the prior distributions were made based on the posterior distributions of the parameters. For the chloroplast markers, five sets of one-sample summary statistics (number of haplotypes, number of segregating sites, mean pairwise differences, number of private segregating sites and mean number of rarest nucleotide at segregating sites) and two sets of two-sample summary statistics (mean of pairwise differences between samples and F_{ST}) were used. For the nuclear markers, four sets of one-sample summary statistics (mean number of alleles, mean genetic diversity, mean size variance and mean Garza-Williamson's M) as well as two sets of two-sample summary statistics (F_{ST} and shared allele distance) were used. The scenarios were compared by simulating 1 000 000 datasets per scenario. The closest 1% of the simulated datasets of the observed data was used to estimate the posterior probabilities of each scenario by applying the logistic regression with a log-it transformation. The confidence of the scenario choice was assessed by estimating type I and type II error rates according to Cornuet *et al.* (2010). To assess the ability of the scenario with the highest posterior probability to simulate datasets similar to the observed data, the deviations of the posterior prediction of the summary statistics from the observed data were assessed by performing a model check based on 6 000 simulated datasets.

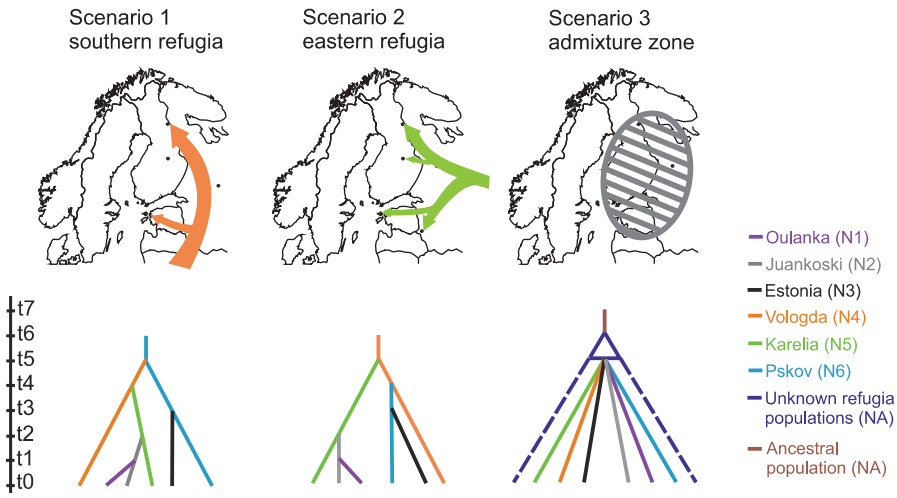

Fig. 4. Graphic representation of the colonisation scenarios studied using DIYABC.

3 Results and discussion

3.1 Viability of small populations

Small populations performed significantly worse compared to large populations, as indicated by the deterministic analysis (Table 1; I). The deterministic growth rate was significantly correlated with the total population size ($R^2 = 0.779$, $p = 0.005$; I) and was significantly lower in small populations ($N < 100$, $\lambda_d = 0.993\text{-}0.994$; I) compared to large populations ($N < 100$, $\lambda_d = 1.000\text{-}1.016$; I) ($F_{1,9} = 51.902$, $p < 0.001$; I). Fecundity was considerably lower in small populations which might explain their low viability. No seedling recruitment was observed during the three years of monitoring in the small populations, except one in Kiutaköngäs SWc (I).

In long-lived plant species, the minor importance of seed production and seedling recruitment on the population dynamics and long term viability is assumed (Franco & Silvertown 2004). However, it has been suggested that both low seed production rates and low recruitment rates limit the persistence of populations of orchid species (Jacquemyn *et al.* 2010, Sletvold *et al.* 2010, Juárez *et al.* 2014). This is especially the case in small and fragmented populations, in which reduced seed production due to lowered fruit set for example, can cause low fecundity rates.

There are numerous ecological and genetic processes that can lead to the reduced seed production and lowered fruit set. In small populations, individuals can fail to form seeds due to the lack of flowering conspecifics or low density, i.e. Allee effect (Groom 1998, Lamont *et al.* 1993, Courchamp *et al.* 1999) or due to the lack of pollinators, i.e. pollination limitation (Sih & Baltus 1987, Ågren 1996). Orchid species which are highly specific in pollinators, are vulnerable to pollination limitation which has been observed in several orchid species (Gijbels *et al.* 2015). However, fruit set was observed in all small populations, indicating that plants did not fail to form seeds (I). In addition, no significant correlation between the fruit set and total population size was observed ($R^2 = 0.339$, $p = 0.308$; I), giving a further indication that pollen limitation did not reduce fecundity in small populations. Similarly, a lack of such correlation has been found in other orchid studies (Calvo & Horvitz 1990, Calvo 1993). However, orchids produce large quantities of seeds within capsules and consequently studying pollen limitation using observations of fruit set cannot exclude the possibility of pollen limitation (Sletvold *et al.* 2012, Jacquemyn & Brys 2015). Additional research, such as pollen

supplementation experiments, could be used to assess the extent of pollen limitation more thoroughly.

Table 1. The demographic parameters of each population obtained from the matrix models and genetic diversity estimates (I).

Population	n	T	N	λ_d	λ_s	P_e	t_{viab}	F	H_e	A_R
Ampumavaara	119	16	139	1.009	0.992	0.036	106	0.051	0.351±0.185	1.841±0.408
Kiutaköngäs N	133	16	133	1.016	0.993	0.084	85	0.082	0.439±0.128	2.084±0.389
Patoköngäs	86	14	145	1.006	0.973	0.934	36	0.028	0.242±0.124	1.598±0.255
Mataraniemi NW	117	3	499	1.000		0.523	72	0.024	0.397±0.206	1.85±0.634
Kiutaköngäs SWc	42	6	42	0.994		1.000		0.020	0.361±0.102	1.747±0.393
Kiutaköngäs b	27	3	27	0.993		1.000		0	0.225±0.099	1.524±0.353
Kiutaköngäs E	27	3	27	0.993		1.000		0	0.378±0.197	1.440±0.550
Kiutaköngäs NWb	26	3	26	0.993		1.000		0	0.256±0.261	1.491±0.694
Kiutaköngäs NWc	6	3	6	0.993		1.000		0	0.300±0.166	1.478±0.443
Kiutaköngäs SWa	9	3	9	0.994		1.000		0	0.505±0.135	1.845±0.714
Kiutaköngäs W	14	3	14	0.993		1.000		0		

Number of monitored individuals per population (n), time of monitoring in years (T), total population size (N), deterministic population growth rate (λ_d), stochastic population growth rate (λs), probability of extinction (P_e), viable time (t_{viab}), fecundity (F), expected heterozygosity (H_e) and allelic richness (A_R).

Pollen limitation and Allee effect do not necessarily result in a lower fruit set as the lack of conspecifics or pollinators can also lead to a higher selfing rate. *E. atrorubens* has been shown to be predominantly allogamous, but no self-incompatibility has been observed (Tałałaj & Brzosko 2008). However, a very low capacity for autonomous selfing has been documented for *E. atrorubens* (Brys & Jacquemyn 2016). In addition, less movement of pollinators among patches and more visitations to flowers of the same plant are often observed in small and sparse

populations compared to large or dense populations (Goverde *et al.* 2002, Grindeland *et al.* 2005). This can lead to a higher rate of geitonogamous self-pollination and a higher probability of mating between related individuals (biparental inbreeding) (Dudash & Fenster 2000). Reduced cross-pollination and increased breeding with relatives can result in inbreeding depression (Ellstrand & Elam 1993, Young *et al.* 1996, Reed & Frankham 2003). Inbred individuals are more homozygous than outbred individuals, increasing the probability of the expression of harmful and deleterious recessive alleles, which can further reduce the fitness through a lowered fecundity and decreased survival (Ellstrand & Elam 1993, Young *et al.* 1996, Reed & Frankham 2003). In addition, the fitness can be reduced due to the loss of heterozygosity advantage (over-dominance). The high costs of inbreeding depression for the seed set and seed viability have been documented in a number of predominantly allogamous orchid species (*Arcolophia cochlearis*; Peter & Johnson 2009, *Gymnadenia conopsea*; Sletvold *et al.* 2012, *E. atrorubens, E. helleborine and E. palustri*; Brys & Jacquemyn 2016). In the studied populations, no correlation between the population size and the level of inbreeding was found, since individual heterozygosity was not significantly lower in small populations when compared to large populations ($F_{1,195} = 0.198$, $p = 0.720$; I) and there was no relationship between the inbreeding coefficient (F_{IS}) and the population size ($R^2 = 0.045$, $p = 0.901$; I). In addition, no indications for a lower survival due to inbreeding depression were found when the capture recapture analyses were used, as the individual survival was not affected by the heterozygosity levels (I). In this study genetic diversity was studied using neutral genetic markers. The levels of genetic diversity obtained do not necessary correlate directly with the fitness of individuals. Further research using selective traits could give further insights on the presence of inbreeding depression.

In orchids, the fecundity has been suggested to be limited by low seedling recruitment rates rather than low fruit set rates (Calvo & Horvitz 1990, Calvo 1993). While orchid seedling recruitment success depends on both seed production and the availability of suitable microhabitats for germination, it has been suggested to be limited by the availability of suitable habitats (Kull 1998). As orchid seeds do not contain enough nutrient reserves to produce autotrophic seedlings, successful germination occurs only in association with appropriate mycorrhizal fungi (Rasmussen 1995). The availability of suitable mycorrhizal fungi is, therefore, expected to be a major factor for successful seedling recruitment and the distribution of orchid populations (Rasmussen 1995, McConrmick & Jacquemyn 2014). Additionally, orchids, including *E. atrorubens*, have been shown to be

highly specific in their association with mycorrhizal fungi during the protocorm stage (Bidartondo et al 2004, McCormick at al. 2004, Shefferson *et al.* 2007, Bidartondo & Read 2008) and this specificity could further constrain the seedling recruitment. Studies have shown that the seedling recruitment success of orchid species decreases with the distance between the mother plant and the seedling (Batty *et al.* 2001, Diez 2007, Waud *et al.* 2016). The biotic and abiotic conditions around adult individuals are therefore expected to be more suitable for seedling germination. A study using qPCR analyses has shown that successful recruitment clustered around the adult orchids, due to a rapid decline in the abundance of mycorrhizal fungi with the distance to adult orchids (Waud *et al.* 2016). On the contrary, seed germination has not been shown to be limited by mycorrhizal availability in orchids as well (Waud *et al.* 2017). Consequently, it can be expected that seedling recruitment success is higher in dense sites due to a higher abundance of mycorrhizal fungi associated with the roots of adult plants and that the availability of suitable microhabitats in small populations is likely to be lower compared to that in large populations.

3.2 Stochasticity

High levels of inter-annual and spatial variation in the demographic parameters were observed in the study populations (I). The temporal and spatial variation was especially noticeable in the seedling recruitment rate. The overall fecundity varied considerably, from 0 to 0.082, between populations and there was also high inter-annual variation, i.e. F = 0–0.393 in large populations (I). Population viability analyses based on stochastic simulations are assumed to be more realistic compared to deterministic models (Shaffer 1981, Bierzychudek 1982, Lande 1988, Menges 2000).

The population growth rates of large populations decreased when stochasticity was included (λ_d = 1.006–1.016, λ_s = 0.973–0.993; I). The low stochastic growth rates indicate a decline in population growth over a long period of time. This was also reflected by the probability of extinction (P_e), which suggested extinctions for all large populations ($P_e > 0.050$), except one (Ampumavaara $P_e = 0.028$), in the next 100 years (I). Stochasticity increases the extinction risk of populations and the substantial impact of stochasticity on population extinctions has been documented by several studies for both animal (Fujiwara & Caswell 2001, Melbourne & Hastings 2008) and plant species (Menges 1990, Damman & Cain 1998). Damman & Cain (1998) showed that the persistence of populations decreased when

stochasticity was included in the models. Furthermore, the minimum viable population size increased remarkably when demographic stochasticity was included in simulations to assess the viability of *Asarum canadense*. A study on the viability of *Pedicularis furbishiae* showed that the estimated persistence decreased when variability was considered (Menges 1990). Knowledge on how stochasticity impacts the viability of species with different life history characteristics is an important aspect of conservation biology (Lande 2002). Deterministic estimates of the population viability such as λ_d and population size reflect the state of the population at a given time and may be inadequate for predicting the future state of the population, as variation in the demographic parameters due to environmental and demographic stochasticity are ignored (Akçakaya 2000). Demographic stochasticity causes random fluctuations in population size and decreases the long-term growth rate (Lande 2002). It is likely that the small populations are not able to buffer for the demographic stochasticity and conservation effort should be focussed on retaining the large, present population sizes.

The impact of stochasticity varied across the large populations. The effect of stochasticity was the highest in population Patoköngäs, reflected by its high probability of extinction ($P_e = 0.934$) and low stochastic population growth rate ($\lambda_s = 0.973$) (I). The stochastic matrix model simulations revealed that an increased population size (N_0 increase) positively affected the persistence of large populations (Fig. 5; I) and that an initial population size of >300 individuals was required for a viable population.

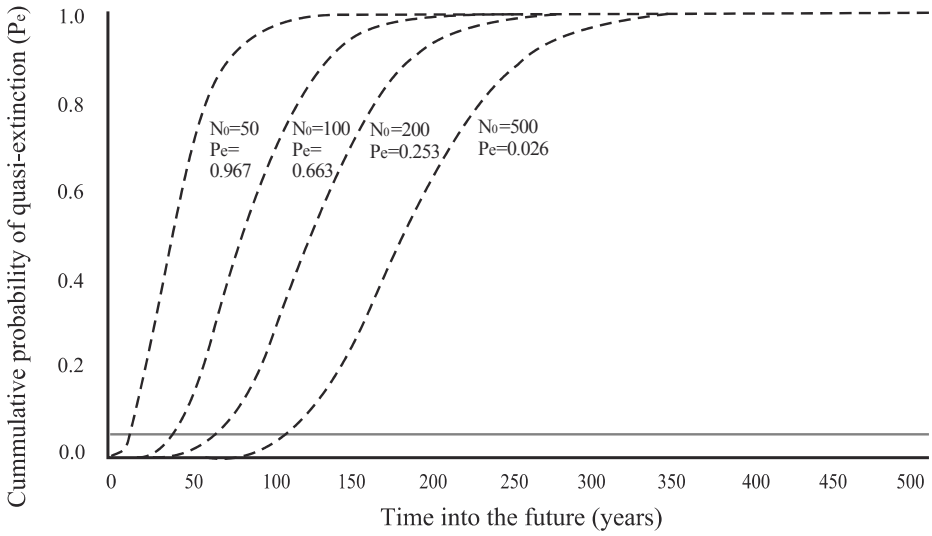

Fig. 5. The probability of extinction (P_e) for different hypothetical initial population sizes (N_0) for the large populations combined (I). The projection was simulated for 500 years into the future. The grey line represents the 5% viability threshold.

3.3 Genetic variation

The level of genetic variation affects the long-term viability of populations, as genetic variation enables populations to adapt to environmental changes (Ellstrand & Elam 1993, Leimu *et al.* 2006, Jump *et al.* 2009, Dierks *et al.* 2012). Small populations often have low levels of genetic variation, as variation is lost through random genetic drift. Orchids are generally assumed to be able to harbour high levels of genetic variation, as the combination of outcrossing reproduction, prolonged dormancy, seedbanks and a long generation time is expected to buffer the loss of genetic variation (Templeton & Levin 1979, Honnay *et al.* 2008) and enhance breeding with unrelated conspecifics (Rees 1996, Jäkäläniemi *et al.* 2011). Indeed, studies on orchid species have reported high levels of genetic variation in terms of heterozygosity (H_E = 0.62–0.81 for *Gymnadenia conopsea*; Gustafsson 2000, H_E = 0.77–0.92 for *Ophrys* sp.; Soliva & Widmer 2003, H_E = 0.510–0.821 for *Caladenia huegelii*; Swarts *et al.* 2008, H_E = 0.355–0.544 for *Cypripedium kentuckiense;* Pandey *et al.* 2015). However, low genetic diversity has been observed as well (H_E = 0.191-0.322 for *Epipactis helleborine*; Ehlers & Pedersen 2000, H_E = 0.000–0.065 for *Cephalanthera longibracteata*; Chung *et al.* 2004).

When compared to other orchid species, *E. atrorubens* has a complex life history, with a long generation time, protocorm stages, associations with fungi and a prolonged dormancy period. It is thus expected to harbour high levels of genetic variation. However, we found low levels of genetic variation in terms of the number of microsatellite alleles, the number of chloroplast haplotypes and variable nucleotide sites (Table 2, 3; III). The observed heterozygosity levels were moderately low, both on regional (mean H_E = 0.358; 0.277–0.511; III) and fine (mean H_E = 0.390; 0.198–0.833; II) spatial scales respectively when compared to other orchid species. Low levels of genetic variation have also been observed in populations of *E. atrorubens* in Italy (Tranchida-Lombardo *et al.* 2011) and Poland (Brzosko *et al.* 2006).

Table 2. Genetic diversity estimates and indicators for past population size changes using nuclear microsatellite loci (III).

Population	N	H_0	H_E	A_R	F_{IS}	P_{HW}	P_W	G-W
Oulanka	100	0.246	0.354	2.324	0.307	0.001	0.016	0.437
Hanko	24	0.341	0.346	1.899	0.005	0.466	0.016	0.436
Juankoski	30	0.294	0.361	2.029	0.197	0.045	0.016	0.483
Estonia	60	0.411	0.381	2.123	-0.080	0.080	0.016	0.451
Karelia	29	0.470	0.416	1.853	-0.133	0.083	0.016	0.357
Psvok	30	0.233	0.277	1.445	-0.027	0.574	0.016	0.397
Vologda	17	0.511	0.401	1.548	-0.289	0.021	0.016	0.556

Number of sampled individuals (N), observed heterozygosity (H_O), expected heterozygosity (H_E), allelic richness (A_R), inbreeding coefficient (F_{IS}), p-value for hardy Weinberg equilibrium (P_{HW}), p-value for Wilcoxon sing rank test (P_W), Garza Williamson index (G-W).

Table 3. Genetic diversity estimates for sequenced chloroplast loci (III).

population	rbcL-accD						$trnK^{UUU}$					
	N	H	S	H_d	π	θ_S	N	H	S	H_d	π	θ_S
Oulanka	81	2	1	0.336	0.0007	0.0004	51	5	3	0.619	0.0017	0.0011
Estonia	56	2	1	0.508	0.0011	0.0005	56	3	2	0.593	0.0017	0.008
Karelia	27	1	0	-	-	-	25	2	1	0.513	0.0009	0.005
Psvol	11	2	1	0.324	0.0007	0.0006	25	3	2	0.347	0.0011	0.0010
Vologdskaya	16	2	1	0.533	0.0011	0.0006	15	3	2	0.686	0.0018	0.0011

Individuals with successful amplification (N), number of haplotypes (H), number of segregating sites (S), haplotype diversity (H_d), nucleotide diversity (π), Watterson's theta (θ_S).

As genetic variation is the basis for evolutionary potential (Ellstrand & Elam 1993, Leimu *et al.* 2006, Jump *et al.* 2009), low levels of genetic variation can have

negative consequences on the long-term viability of populations. However, the effect of genetic diversity on the long-term viability has been rarely studied in natural populations. In the studied populations, the allelic richness was significantly correlated with the deterministic growth rate ($R^2 = 0.718$, $p = 0.019$; I). However, no significant correlation was found between the allelic richness and the population size ($R_2 = 0.433$, $p = 0.184$; I) nor was there any found between the heterozygosity and population size ($R^2 = 0.025$, $p = 0.946$; I) and the deterministic growth rate ($R^2 = 0.296$, $p = 0.406$; I). The population growth rate probably represents long-term viability better than the population size, as the population size represents the momentary state of the population. As genetic diversity (e.g. allelic richness) reflects the evolutionary potential of the population, the effect of genetic variation is likely to be more pronounced in the long-term and allelic richness probably correlates better with the long-term viability. Heterozygosity on the other hand, is likely to affect the short-term viability more. The significant correlation between the allelic richness and population growth rate highlights the importance of incorporating the information on genetic viability into population viability assessments. Further research assessing genetic diversity using non-neutral genetic markers could give further insights on the role of genetic diversity in the fitness of populations.

3.4 Genetic differentiation and gene flow

Gene flow in plants can occur through seed and pollen dispersal. In orchids, seeds are dispersed by wind and pollen by pollinators. High levels of gene flow are expected in orchid species as they typically produce large numbers of light wind-dispersed seeds that are assumed to be dispersed over long distances (Murren & Ellison 1998, Arditti & Ghani 2000). Such high levels of seed dispersal are expected to result in low population genetic differentiation. Indeed, the reported genetic differentiation values for orchid species are among the lowest in plant families (Hamrick & Godt 1996).

A significant overall population genetic differentiation was observed on the regional spatial scale, especially for the cpDNA (nuclear DNA $F_{ST} = 0.071$, cpDNA $\Phi_{ST} = 0.236$; III). This can be explained by the long distances (mean distance 563 km, range 105–963 km) between the regions, which prevent gene flow between populations. On the fine spatial scale, the short distances between the populations (mean distance = 7.780 km, range 0.062–25.898 km) should allow sufficient levels of gene flow to prevent the occurrence of genetic differentiation through drift.

However, significant levels of genetic differentiation were observed on the fine spatial scale as well (nuclear DNA F_{ST} = 0.110, cpDNA F_{ST} = 0.543–0.545; II). The genetic population differentiation on the fine spatial scale was further highlighted by STRUCTURE and IBD analyses. The Bayesian structure analysis indicated the presence of two clusters based on the ΔK value and additional sub-structuring within the two clusters was highlighted by a second highest ΔK value for K = 5 (see Fig. 3 in II). The recurring occurrence of assignment probabilities of a particular cluster in populations located close to each other indicates the occurrence of IBD, due to dispersal barriers or due to restricted dispersal (Fig. 5; II). Indeed, indications of IBD were observed as significant negative correlations of the mean kinship coefficient and the natural logarithm of the geographic distances for both the nuclear loci (R^2 = 0.001, p = 0.002; II) and the chloroplast microsatellite locus (R^2 = 0.002, p = 0.029; II) (Fig. 6; II).

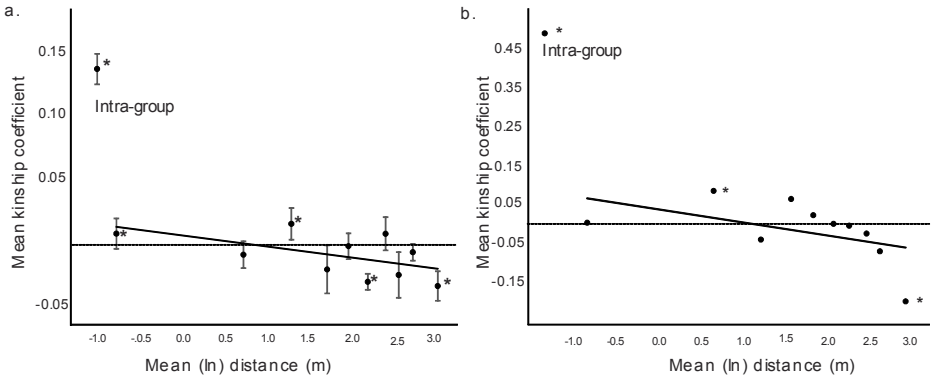

Fig. 6. The mean kinship coefficient within groups (Intra-group), the correlation of the mean kinship coefficient of individual pairs between groups of ten different distance classes and the natural logarithm of the geographical distances for (a) the nuclear microsatellite loci and (b) the trnK microsatellite locus. Mean kinship coefficients between groups which are significantly different from zero are marked with an asterisk (II).

The occurrence of genetic population differentiation and IBD on the fine spatial scale indicates lower levels of gene flow than expected. Low gene flow was further highlighted by the assignment analysis, which indicated the presence of only 20 first generation migrants (2.72%) among the 735 studied individuals (II). While this cannot be interpreted as an exact number, the low number strongly indicates low levels of gene flow among the sampled sub-populations. Spatial genetic

structuring has been found for other orchid species as well, which was often attributed to limited seed dispersal (Chung *et al.* 2004, Chung *et al.* 2005, Jacquemyn *et al.* 2006, Wallace 2006). Orchids are expected to have a predominately seed mediated gene flow and only a moderate level of pollen mediated gene flow among populations. As the small, wind-dispersed orchid seeds are typically produced in large numbers, they are assumed to have an abundant dispersal over long distances (Murren & Ellison 1998, Arditti & Ghani 2000, Hamrick & Trapnell 2011). Restricted pollen mediated gene flow among populations on the other hand, is expected, as it is restricted by the foraging range of pollinators (Levin 1981). *E. atrorubens* is pollinated by bumblebees and wasps (Brzosko *et al.* 2006, Tałałaj & Brzosko 2008, Jakubska-Busse & Kadej 2011), that have relatively short foraging ranges (Walther-Hellwig & Frankl 2000, Osborne *et al.* 2008).

The population genetic differentiation analyses indicated that the levels of gene flow by seeds is lower than expected. The recurrent detection of large levels of chloroplast genetic differentiation, with pairwise F_{ST}-values up to 1 (II), even between adjacent populations, indicates restricted seed dispersal, as chloroplast DNA is only dispersed by seeds. On the other hand, the moderate nuclear differentiation levels indicate higher levels of pollen mediated gene flow. The significantly high m_p/m_s ratio (7.68-7.74; II) indicates that the contribution of pollen mediated gene flow to the total gene flow is significantly higher than seed mediated gene flow. Such high m_p/m_s ratios have been observed for several other orchid species as well (14.25 for *Epidendrum fulgens*; Pinheiro *et al.* 2011, 4.25 for *Brassavola nodosa*; Trapnell *et al.* 2013, 46.3 for *Epidendrum firmum*; Kartzinel *et al.* 2013). These results indicate that the seed mediated gene flow is lower than expected. Low seed dispersal distances, often limited to a few metres from the mother plant, have been observed in several orchid studies (Murren & Ellison 1998, Machon *et al.* 2003, Jacquemyn *et al.* 2007, Chung & Nason 2007). Low germination success has been suggested to be the cause of a low number of effective long distance dispersal events in orchids, rather than the absence of long distance seed dispersal. Indeed, studies have shown that seed dispersal distances are much larger than the average distance between the adult plants and recruits in orchid species (Jacquemyn *et al.* 2007, Jacquemyn *et al.* 2009). Seed germination success depends strongly on the abiotic and biotic habitat conditions and has been shown to decrease with the distance between the mother plants and seedlings in orchids (Batty *et al.* 2001, Diez 2007, Waud *et al.* 2016).

3.5 Historical processes

The present population genetic differentiation and variation patterns are governed by various factors, including present population dynamics (Ennos 1994), local selection (Linhart & Grant 1996) and historical events (Kalisz *et al.* 2001). Historical processes that have strong implications for the present geographic and population genetic differentiation patterns include past population disturbances and population distributions such as range expansions, isolation of populations and population size changes. In this study past population changes were studied using a set of various tests and indications of the decrease in population size were found by the nuclear markers in all studied regions (III).

This study further examined the post-glacial colonisation of the Finnish populations of *E. atrorubens*. Population contractions during the LGM and bottlenecks during the range expansions could have left their mark on the population genetic patterns (Hewitt 1999, Hewitt 2004, Arenas *et al.* 2012). The coalescent-based approximate Bayesian computations indicated that a scenario with a post-glacial colonisation of the studied regions from a single southern refugium is not sufficient to explain the current phylogeographic patterns. Instead, the admixture scenario (Scenario 3 in Fig. 4) had the best fit to the observed genetic patterns with a high confidence level (III). This suggests that the studied regions were founded by multiple colonisation routes. Scandinavia has also been shown to be a contact zone of different colonisation routes for many other species (Taberlet *et al.* 1995, Jaarola & Tegelström 1995, Taberlet *et al.* 1998, Kyrkjeeide *et al.* 2014).

Range expansions originating from refugia after the LGM would be accompanied with repeated founder events. Such repeated founder events are expected to result in lower genetic variation of populations in areas previously covered with continental ice, as compared to populations located in the refugia (Hewitt 1996, Provan & Bennet 2008, Pellissier *et al.* 2016). The post-glacial colonisation history is thus expected to leave strong geographical patterns in genetic variation. However, in the studied regions no clear geographical pattern in the present genetic variation was found. It has been shown that the remaining levels of genetic diversity after range shifts depend on the speed of the shifts, and a smaller amount of genetic variation is expected to be lost with a slow expansion (Cobben *et al.* 2011, Arenas *et al.* 2012). The lack of a clear geographical pattern in the genetic variation and the absence of IBD (nuclear DNA $r^2 < 0.001$, $p = 0.267$; III) on the large spatial scale indicates that the post-glacial expansion might have been a slow process. The colonisation abilities of plant species may be determined

by the reproduction mode and growth form. A long generation time coupled with a reproduction associated with pollinators could cause low colonisation rates. Individuals of *E. atrorubens* reach the reproductive stage after several years after germination (mean age of reproduction is 7.1 years, range 4–11years; I). The colonisation of new populations is considered to be slower in animal pollinated species compared to wind pollinated species (Govindaraju 1988), due to a lower reproductive output. However, while pollen dispersal can only contribute to gene flow among populations, seed dispersal is the only dispersal mechanism that contributes to the colonisation of new populations. Consequently, the slow post glacial expansion could be explained by the infrequent long-distance seed dispersal events, as indicated by the results of the present genetic structure on the fine spatial scale.

4 Conclusion

This study assessed the viability of *E. atrorubens* populations and found low persistence in small populations, low levels of genetic diversity and spatial population structuring.

Small populations of *E. atrorubens* have remarkably low population growth rates, predominantly due to a low germination success. This low germination success is more likely due to the lack of suitable microhabitats and mycorrhizal fungi in small populations, than ecological or genetic processes such as inbreeding depression, pollen limitation or pollinator deficit. Suitable micro-habitats are expected to be more abundant in large and dense populations, as indicated by the decrease in abundance of mycorrhizal fungi from adult orchid plants (Waud *et al.* 2016) for example.

Despite the expected abundant long distance seed dispersal of orchids, low seed mediated gene flow was found among the studied populations, as indicated by low but significant spatial genetic structuring and a significantly high ratio of pollen to seed gene flow. In addition to high expected levels of gene flow, orchids are assumed to harbour high levels of genetic variation due to their high expected levels of seed dispersal and life history characteristics such as outcrossing, seed banks, prolonged dormancy and long generation time. However, this study showed opposite results, i.e. low levels of genetic variation. Being the evolutionary substrate for adaptations to new environmental conditions, low levels of genetic variation can have negative implications for the populations in changing environments. While there is an extensive theoretical background on the effects of genetic variation on the viability of species, there are only few studies conducted on natural populations. Although this study did not find indications for the negative effect of inbreeding on the population viability or the survival of individuals, a significant correlation was found between the allelic richness and population growth rate. Here, the genetic variation was studied using neutral markers, but future research using traits under selection is needed to study the adaptive abilities of the species.

In addition to an adaptive response, species and populations can avoid extinctions by responding to the changing environmental conditions, such as climate change, by shifting their ranges to more suitable habitats (Parmesan & Yohe 2003, Root *et al.* 2003, Corlett & Westcott 2013). Therefore, the colonisation ability of the species can have strong implications for its viability. However, the colonisation speed required to keep up with the changing climate is much faster

than previously thought and may be faster than the duration of a generation in perennial plant species (Corlett & Westcott 2013, VanDerWal *et al.* 2013). Low rates of current seed dispersal and indications of the occurrence of slow range expansion in the past indicate the lack of fast colonisation abilities of *E. atrorubens*. Slow colonisation rates can have a strong negative impact on species viability, as the species may not be able to keep up with the rapid climate change.

The combination of low genetic variation, low seedling recruitment rate and low seed dispersal rates can have adverse consequences on the viability of the species and it predicts that the ability of *E. atrorubens* to cope with the environmental change is very limited. Besides the capability of species to colonize new suitable habitats, the ability to reach these areas can be further restricted by habitat loss and fragmentation. The ability to persist in new areas can be hampered by competition with resident species which may be more adapted to the conditions in the new area, and with new invasive species (Thomas *et al.* 2004, Corlett & Westcott 2013).

To conclude, this study shows that seed dispersal and seedling recruitment are important processes limiting the viability of populations. The low seed germination rate, probably coupled with an insufficient amount of suitable micro-habitats in small populations, potentially lowers seedling recruitment and consequently, effective seed dispersal. Seedling recruitment, therefore, affects the population dynamics, genetic diversity and spatial genetic structure of plant populations. Suitable habitat conditions are likely to be more abundant in large and dense populations and therefore the management of large populations should be the focus for further conservation actions. The importance of maintaining large populations was further highlighted by the effect of stochasticity on the viability of populations. In conclusion, large populations are needed as a buffer for the negative impact of stochasticity, maintenance of genetic variation and for enabling sufficient seedling recruitment.

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Original publications

I Hens H, Pakanen VM, Jäkäläniemi A, Tuomi J, Kvist L (2017) Low population viability in small endangered orchid populations: genetic variation, seedling recruitment and stochasticity. Biol Conserv 210: 174–183.

II Hens H, Jäkäläniemi A, Kvist L (2015) Restricted seed-mediated gene flow, fine-scale spatial genetic structure and low genetic diversity in a regionally endangered orchid. (Manuscript)

III Hens H, Jäkäläniemi A,Tali K, Efimov P, Kravchenko AV, Kvist L (2016) Genetic structure of a regionally endangered orchid, the dark red helleborine (*Epipactis atrorubens*) at the edge of its distribution. Genetica 145:209–221.

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