Low population viability in small endangered orchid populations: genetic variation, seedling recruitment and stochasticity

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Abstract

There are only few studies that use both demographic and genetic data to assess population viability of plant species. We combined genetic and demographic data from 11 endangered perennial orchid populations of varying size in order to reveal determinants of viability. Small populations had substantially lower viability compared to large populations. Seedling recruitment rates were remarkably lower in small populations; this was not due to pollination limitation or inbreeding depression because the fruit set and heterozygosity were not correlated with population size, suggesting that there may be differences in successful germination. Low recruitment resulted in significantly lower predicted population growth rates in small populations. The impact of stochasticity on viability varied among populations and stochastic simulations indicated that only one large population was viable, whereas all the other large populations were predicted to go extinct within decades. While there was a positive correlation between the deterministic population growth rate and allelic richness, we did not find any other correlations between genetic variation and fitness or population size. The study populations are likely remnant populations of a once large meta-population that decreased in size due to unfavourable environmental conditions. Management should focus on the maintenance of large population size, which is needed to avoid negative consequences of stochasticity and to enhance seedling recruitment rates.

Keywords: *Epipactis atrorubens*; genetic diversity; mark-recapture model; population growth rate; population viability; transition matrix

Highlights

-The viability of an endangered orchid was studied using demographic and genetic data.
-Small populations had considerably lower viability compared to large populations.
-Low seedling recruitment rates result in low viability of small populations.
-Allelic richness correlated positively with the deterministic population growth rate.

1. Introduction

Endangered species often consist of small and fragmented populations, which are vulnerable to extinction due to environmental and demographic stochasticity (Lande 1988; Shaffer 1981). In addition, small populations suffer from genetic stochasticity caused by genetic drift and inbreeding (Young et al. 1996). A higher probability to mate with related individuals or elevated selfing rates lead to increased levels of homozygosity, which may result in the expression of harmful and deleterious recessive alleles, and consequently reduced fecundity and survival of individuals in small populations (Ellstrand and Elam 1993; Reed and Frankham 2003; Young et al. 1996). Additionally, random genetic drift is expected to lead to a reduced genetic diversity in small populations. Reduced genetic variation can constrain the evolutionary potential, and thus lower long-term viability of plant populations especially in rapidly changing environments (Ellstrand and Elam 1993; Young et al. 1996). While it is recognised that genetic variation is important for long-term evolutionary processes, its short-term influence on the extinction risk has been questioned (Caro and Laurenson 1994; Caughley 1994; Lande 1988). It has been suggested that genetic factors only act after populations have already undergone significant declines in population size. However, there is extensive evidence for the influence of genetic factors on the extinction risk (Angeloni et al. 2011; Hedrick and Kalinowski 2000; Reed and Frankham 2003). For example, genetic erosion has been observed in small fragmented populations (Aguilar et al. 2008; Honnay and Jacquemyn 2007; Leimu et al. 2006), and inbreeding has been shown to decrease fitness in small populations (Oostermeijer et al. 1994). Levels of populations' genetic diversity are thus expected to correlate with both the population size and the long-term population viability.

In addition, ecological processes may reduce the population size further, after the population sizes are reduced due to deterministic processes. Such ecological processes include a reduced pollination due to the lack of conspecifics or low density (Allee effect; Courchamp et al. 1999; Groom 1998; Lamont et al. 1993) or due to the failure of small populations to attract pollinators (pollination limitation; Sih and Baltus 1987; Ågren 1996), may reduce the population size further.

A key for undertaking effective conservation actions is to understand the importance of genetic variation, stochasticity, demographic parameters and deterministic population dynamics on the performance of populations. To date, mostly demographic parameters have been used to gain knowledge on the population viability (Reed et al. 2002) and only few studies have incorporated both demographic and genetic parameters (e.g. Menges and Dolan 1998; Oostermeijer et al. 2003; Reed et al. 2002; Schmidt and Jensen 2000). Furthermore, studies often ignore important life history factors, such as dormancy, seed banks or clonal reproduction (Zeigler et al. 2013).

The family of Orchidaceae has the highest proportion of endangered species of all plant families (Swarts and Dixon 2009). Orchids typically depend on biotic interactions for reproduction and germination, and are specialised in specific mycorrhizal fungi (Rasmussen 1995) and pollinators (Micheneau et al. 2009). In addition, orchids tend to be specialised in particular abiotic habitat conditions. High specialisation makes orchids vulnerable to the negative consequences of changing environmental conditions, as these inter-specific interactions can be altered, leading to decreased population sizes and, eventually local extinctions (Fay et al. 2015; Micheneau et al. 2009). Conservation of orchid species is challenging, because these interactions can be altered in many different ways by environmental factors, such as climate change and habitat fragmentation (Fay et al. 2015). Understanding the consequences of declining population sizes and environmental changes on the demography of orchids is thus important for the conservation of these species (Swarts and Dixon 2009).

In this study, we combined genetic and demographic data to examine population viability of an endangered perennial orchid, the dark red helleborine (*Epipactis atrorubens* (Hoffm. Ex Bernh.) Besser). We used data collected from eleven populations of varying size to examine whether there was variation in the performance of different populations. The data enabled the examination of the

effect of population size, and associated processes (demography, stochasticity and genetics) affecting population viability. We first estimated demographic parameters from long-term data (three to sixteen years). We then used matrix population models to estimate population growth rates, identified the most important life stages for population growth rates, and used them in population viability analysis to estimate extinction risks and the effect of stochasticity. Second, we genotyped individuals based on six nuclear microsatellite markers, and quantified different measures of genetic variation. We then assessed the effect of genetic diversity on population viability by testing for correlations between genetic variation and viability measures, which included application of capture-recapture methods for modelling the effect of individual heterozygosity on survival.

2. Material and methods

2.1 Study species

E. atrorubens is a perennial orchid, with a distribution extending from Europe until the Ural Mountains in Asia and western Siberia (Efimov 2004; Tuulik 1998). In Finland, its distribution is patchy, with populations occurring in only three regions, separated by at least 350 km. The species is currently categorized as vulnerable in Finland according to the IUCN red list criteria (Rassi et al. 2010). Their habitat consists mainly of rocky slopes and shorelines, and it is characterized by limestone or dolomite. It has a predominantly allogamous reproduction, where pollination occurs by both wasps and bumblebees (Brzosko et al. 2006; Jakubska-Busse and Kadej 2011; Talalaj and Brzosko 2008). *E. atrorubens* has been shown to be predominantly allogamous, but no self-incompatibility has been observed (Talalaj and Brzosko 2008). Individuals live in close association with mycorrhiza and the life-cycle is characterized by a protocorm life stage, overwintering as an underground rhizome and the ability to go into vegetative dormancy for several years (Jäkäläniemi et al. 2011). Its nectar producing flowers bloom at the end of July and capsules ripen in August. Individual plants can ramify from the single root neck just above the ground surface and consequently big plants can have several shoots (1-28 shoots). No indications of vegetative reproduction have been found for this species in the studied populations based on herbarium and field collections (own observations).

2.2 Study area and sampling

This study was carried out in the Oulanka National Park in Kuusamo, Northern Finland (66° 22’ 27” N, 29°13” E). This region houses the northernmost populations (62 populations) of *E. atrorubens* in Finland. These populations are fragmented and genetically differentiated within the studied area (Hens et al. 2017). Three populations were monitored since 2000 or 2002 (for 16 and 14 years, respectively), as being part of a long-term monitoring project. As these populations were few and consisted of only large populations (> 170 individuals), additional and smaller populations were included and monitored since 2009 or 2013 (for six and three years, respectively; Fig. 1; Table 1). From each population, 1-32 leaf samples were collected during 2011-2012, depending on the population size. One leaf was taken from each individual and frozen at -20ºC (Permits: LAPELY/348/07.01/2011, POPELY/568/07.01/2011). To ensure that the samples represent genetically distinct individuals, only one leaf was taken from groups of shoots and samples were taken from well separated individuals.

2.3 Monitoring

In the four large populations (Kiutaköngäs N and Ampumavaara, Patoköngäs, Mataraniemi), individuals were marked and monitored in subsequent years in permanent 10 m x 10 m plots. The plots included different portions of the total population (Kiutaköngäs N 100%, Ampumavaara 80%,

Patoköngäs 46%, Mataraniemi <25%). In the seven small populations, all individuals were monitored. The individuals were tagged using numbered plastic tags in 2000 (Kiutaköngäs N and Ampumavaara), 2002 (Patoköngäs), 2010 (Kiutaköngäs SWa) and 2013 (Kiutaköngäs b, Kiutaköngäs E, Kiutaköngäs NWb, Kiutaköngäs NWc, Kiutaköngäs W, Kiutaköngäs SWc and Mataraniemi) and they were monitored yearly during the flowering time in early August. Each year, all newly found individuals were tagged. The recorded parameters included the number of shoots, number of flowers, number of capsules and the height of the plant for each individual.

2.4 Genetic analyses

2.4.1 Molecular methods

DNA was extracted using a modified 2x cetyltrimethylammonium bromide (CTAB) protocol (Doyle 1987). Six nuclear microsatellite loci were amplified to assess genetic variation (Tranchida-Lombardo et al. 2008; Minasiewicz and Znaniecka 2014) (Table A.1). The PCR amplifications were carried out in a reaction volume of 10 µl, containing 1 x reaction buffer, 0.2 mM of dNTP, 1 µM (SW2-185, SW1-120, SW1-78, SW1-76) or 0.5 µM (SW2-152 and Ccal25) of each primer, 2.0 mM (SW2-185, SW2-152 and Ccal25) or 3.0 mM (SW1-120, SW1-78 and SW1-76) of $MgCl_2$, 0.3 units of Biotools polymerase (B&M Laboratories, Madrid, Spain) and 5 – 20 ng of template DNA. The PCR conditions are given in Table A.2. The PCR products were run with an ABI PRISM 3730 automatic sequencer (Applied Biosystems) and the loci were scored with GeneMapper v. 5.0. In order to quantify genotyping errors due to scoring errors and/or allelic dropouts, the mean error rate per allele and per locus was calculated (Pompanon et al. 2005), based on randomly chosen samples (at least 6% of the sample size) that were genotyped a second time.

2.4.2 Genetic analyses

Micro-checker (Van Oosterhout et al. 2004) was used to quantify genotyping errors due to scoring errors, allele dropouts and null-alleles. Deviations from linkage equilibrium were tested in GENEPOP 4 (Raymond and Rousset 1995; Rousset 2008) within the sample populations. Allele frequencies were calculated with FSTAT v2.9.3.2 (Goudet 2001). The observed (H_O) and expected (H_E) heterozygosities were calculated in ARLEQUIN v3.5 (Excoffier and Lischer 2010). The inbreeding coefficient (F_{IS}) was calculated and the significance of heterozygote excess or deficit was tested using 10000 permutations in FSTAT v2.9.3.2 (Goudet 2001). The allelic richness (A_R corrected for sample size using the rarefaction method) was calculated in FSTAT v2.9.3.2 (Goudet 2001). Individual heterozygosities were calculated as the heterozygosity weighted by locus (Aparicio et al. 2006) using the IR macroN3 in Microsoft Excel (Amos 2005). As only one individual was sampled from population Kiutaköngäs NWc, no genetic diversity estimates were calculated for this population.

2.5 Matrix analyses

2.5.1 Matrix construction

Stage-based or Lefkovitch matrices (Lefkovitch 1965) were constructed for all individual populations using a life cycle graph (Fig. 2). Individuals were classified into eight life stages. Newly emerged individuals that were smaller than 3 cm and had a maximum of two leaves, were classified as seedlings (s). Vegetative individuals (v) had only one vegetative shoot (≥3 cm) and were not observed flowering in the previous years. A mature vegetative individual (mv) was a plant with more than one vegetative shoot and no fertile shoots or a plant with no fertile shoots that flowered in the previous years. A fertile individual (f) was classified as a plant with at least one fertile shoot.

E. atrorubens can be dormant for one or several consecutive years (Jäkäläniemi et al. 2011). Consequently, mortality rates cannot be obtained from short-time data and dormant individuals

cannot be distinguished from dead individuals based solely on above ground observations. In orchid studies, a plant is often classified as dead if it fails to emerge for more than three years (Hutchings 1987), however, longer periods of dormancy have been documented (Shefferson 2009). In this study, dormancy has been observed for up to 13 years (see results). Consequently, individuals can be incorrectly classified as dead when not observed in the last four years of the monitoring period. Nonetheless, as dormancy lasted on average less than three years (see results), errors due to this were considered minimal. Thus, an individual not emerging for more than three years was considered dead. Consequently, the individuals that did not emerge during the last three years or less, could not be classified, as they could be either dead or dormant. Due to this limitation, it was not possible to estimate mortality rates in populations that were monitored for only three years. Therefore, the average mortality rates estimated from populations that were monitored for longer periods were applied for these populations. We assumed that if a plant is classified as dead, it has died during the first year belowground (Instant death *sensu*, Alahuhta et al. 2017), consequently the mortality rates for dormant stages were zero. The estimates of population growth rates or the elasticity values of transitions are not dependent on whether the individual dies at once or after a period in dormancy (Slow death, Alahuhta et al. 2017). The dormant stage was defined when a previously observed individual had no visible above ground tissues during a certain period but re-emerged later. As the life stage before dormancy has an effect on the performance of the individual during and after reappearance (Swarts and Dixon 2009), four dormancy stages were used [dormant seedling (ds), dormant vegetative (dv), dormant mature vegetative (dmv) and dormant fertile (df)] depending on the life stage before dormancy.

In orchids, individuals can stay in an underground protocorm stage for several years, before seedlings emergence. Due to the possibility of existing seed banks and dormant protocorm stages, for variable periods of time, the time between the seed formation and the recruitment is unknown. Therefore, there is a time lag between seed formation and recruitment. Due to restrictions on excavation in these protected habitats, exact knowledge on this is lacking, but a previous study has shown that recruitment can occur two years after the seed production (Bidartondo and Read 2008). In order to find the length of the time lag, we searched for the best model to predict the number of seedlings based on the number of fertile plants at different preceding time periods (i.e. using different time lags). This was done using a generalized linear model, with a negative binomial distribution and a logit link function. The AICc decreased with increasing time lag (Table A.3). Due to the absence of a clear model and the impossibility to know the exact time lag, recruitment as a proxy for fecundity in year t was calculated as the number of seedlings in year t divided by the average number of fertile plants before year t over the monitored years. Transition probabilities between the protocorm stages and from protocorm to small vegetative were set to 1. To test for density dependence for the recruitment of new individuals, a Pearson product-moment correlation between the number of seedlings and number of above ground plants was used (Ampumavaara R^2 = -0.0731, p = 0.788; Kiutaköngäs N, R^2 =-0.369, p = 0.160; Patoköngäs, R^2= 0.0003, p = 0.999). Due to the absence of a significant correlation, no density dependence in recruitment was included in the matrix.

2.5.2 Deterministic matrix models

The census data were used to construct fifteen, thirteen, five or two annual transition matrices per population for the populations monitored for sixteen, fourteen, six and three years, respectively. The annual transition matrices were used to produce a mean population matrix. In the small populations, several transitions were not observed and set to zero.

The finite rate of population growth (λ_d, hereafter the deterministic growth rate) and elasticities (e_{ij}) were calculated for all matrices using the PopTools package v3.2 (Hood 2010) in Microsoft Excel. The population growth rate (λ_d) is the dominant eigenvalue of the transition matrix and was used to assess the performance of a population (Caswell 2001). Elasticities were estimated to determine the relative importance of individual transition rates on population growth (Caswell

2001; Silvertown et al. 1993). The elasticities of the matrix elements were combined into three transition types; growth (transition to the next stage), stasis (individual that stays in the same stage) and recruitment and plotted on a demographic triangle (G-L-F triangle) to analyze the effect of the transition type on λ (Silvertown et al. 1993, 1996). As *E. atrorubens* can go from a fertile to a mature vegetative stage, a backwards transition (regression) is possible. This transition was included in the stasis type for simplicity (Bell et al. 2003; Silvertown et al. 1996).

2.5.3 Stochastic matrix models

In addition to the deterministic matrix models, stochastic matrix models were used to assess the population viability. Due to the short monitoring periods and consequently missing data on many transitions in the individual transition matrices of the small populations and Mataraniemi, stochastic matrix models were not applied on these populations. The stochastic growth rate (λ_S; Morris and Doak 2002) and the probability of extinction (P_e; Morris and Doak 2002) were calculated using the POPBIO v3.2 package (Stubben and Milligan 2007) in R. The annual population transition matrix models were used to calculate the probability of extinction (P_e). Matrices were selected at random with replacement (each matrix had an equal probability of selection). Simulations were performed for 500 years into the future for ten runs of 5000 simulations. The population sizes from 2012 were used as the starting population vectors (N_0), as it was the latest year, when all stages could be defined, including the dormant stages. In the large populations, all individuals inside and outside the 10 m x 10 m plots were counted 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; Table A.4). The extinction risk was defined based on the simulations 100 years into the future. A population was defined as functionally extinct if it contained less than 25 individuals. A population was defined as viable if its probability for extinction was smaller than 5% over the next 100 years (Menges 1990). In order to study the effect of population size on population viability, all the annual population transition matrices of the large populations (N>100) were combined to assess the population extinction probability as described above. Population sizes of 50, 100, 200 and 500 were used as N_0 with the average stage distribution of the large populations.

2.6 Correlations

Pearson product-moment correlation tests were conducted to test for correlations between population size and the fruit set (percentage of flowers that set fruit), population growth rate (λ_d) and recruitment. In addition, we tested for the influence of the genetic variation on population viability by calculating the correlations between measurements of genetic variation (A_R, F_{IS} and H_E) and the population growth rate (λ_d) and the population size. One-way ANOVA was used to test differences in the proportion of fertile individuals between small and large populations, the effect of population size (small population vs. large population) on recruitment and the deterministic growth rate (λ_d) and the impact of the varying monitoring years of the different populations. The analyses of the large populations were limited to the last three monitoring years and the recruitment and the deterministic growth rate (λ_d) were calculated. Further, one-way ANOVA was used to test for the difference in individual heterozygosity between young (defined here as individuals that had not been observed to flower) and old individuals (individuals that had been observed in the flowering stage during at least one year).

2.7 Mark-recapture analyses

Dormancy affects the likelihood of observing individuals, and may bias mortality estimates. Therefore, the data from the three large populations (Kiutaköngäs N, Ampumavaara and Patoköngäs) were further analysed using mark-recapture models that explicitly consider recapture probability when estimating survival (Lebreton et al. 1992). The mark-recapture analyses were conducted in MARK v8.0 (White and Burnham 1999). Recapture-probability (p) can be used to estimate

probability of being dormant as 1-p (Shefferson et al. 2001). Because the different stages are likely to have different survival rates, multi-state models were used to estimate survival to year t+1 given being in a certain stage in year t. The model included three populations and four stages, survival of the stages (phi), recapture probabilities (p) of the stages and transition (M) probabilities between stages, Phi(pop+stage) p(pop+stage) M(pop+stage). We fixed the following probabilities to zero: recapture probability of a seedling, transitions from larger 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 very large compared to the amount of data. Hence, goodness of fit was examined using the median c-hat approach, which suggested no overdispersion ($\hat{c}$ = 0.97, 95% CI 0.79 – 1.14). Note that this model did not explicitly consider the probability of moving from an above ground stage to a dormant stage or *vice versa*. These survival (mortality) estimates were compared to those estimated with the traditional approach.

We used data from individuals that were genotyped with microsatellites to examine whether survival is affected by individual heterozygosity. Sampled individuals included only vegetative, mature vegetative and fertile individuals at the time of sampling and the populations were pooled. The global model with only stage-dependent model parameters [Phi(stage) p(stage) M(stage)] was compared to models where survival of different stages were constrained with an individual heterozygosity value. The models were compared using the Akaike's information criterion corrected for small sample size (AICc). A difference of two AICc units was considered to infer real support for a variable (Burnham and Anderson 2002). The goodness of fit was examined using the median c-hat approach, which suggested no overdispersion ($\hat{c}$ = 0.96, 95% CI 0.87 – 1.04).

3. Results

3.1 Demographic rates

The age (number of years after seedling germination) at which maturity was reached (first year of flowering) varied greatly between individuals from four to eleven years, and was on average 7.4 years (± 2.29 SD). Some individuals did not reach maturity within 16 years. The length of the dormant stage varied from one to thirteen years and was on average 1.34 years (Fig. 3) over all the life-history stages. On average, 16.3 % of the total population remained underground in a dormant stage (calculated from populations monitored during 2007-2012). The number of individuals in each life stage varied among years in each population (Table A.5). The proportion of fertile individuals was larger in the small populations compared to the large populations ($F_{1,9}$ = 7.534 , p = 0.023). The fruit set rate did not increase significantly with population size (R^2 = 0.339, p = 0.308). Recruitment was low in all populations (0-0.086; Table 1) and significantly lower in the small populations compared to the large populations ($F_{1,9}$ = 17.453, p = 0.002). When the analyses were limited to the last three monitoring years, the recruitment of Patoköngäs decreased as no seedlings were observed during the last three monitoring years. As a consequence, the difference in recruitment was not significantly lower in the small populations ($F_{1,9}$ = 2.725, p = 0.133). In the large populations, the annual number of seedlings varied from 0 to 14 (corresponding recruitments = 0 – 0.393; Table A.5). Fruitset was observed in all populations and varied across years (Table A.5). Survival rates for seedlings (average across populations 0.82) showed variation among populations being the lowest in Patoköngäs (Table 2). The average survival was higher in other stages (vegetative = 0.97, mature vegetative = 0.99, fertile = 0.99; Table 2). The population specific matrices are given in Table A.6.

The survival rates estimated with the multi-state model showed the same trend but were lower than those estimated with the matrix approach (Table 2). The probabilities of being dormant (1-recapture rate) were 0.27-0.30 for vegetative, 0.29-0.51 for mature vegetative and 0.0 to 0.01 for fertile individuals in different populations. The probabilities of moving from vegetative stage to mature stage were low (from 0.014 to 0.029). The probabilities of moving from vegetative stage to

fertile stage were 0.075-0.126, while the probabilities of moving from mature vegetative to fertile stage were 0.295-0.389. The probability of moving from fertile to mature vegetative stage ranged between 0.297-0.516.

Survival was not affected by individual heterozygosity. The reduced model was more supported than any model constraining survival with individual heterozygosity (range in ΔAICc = 0.02-2.2), suggesting that the individual heterozygosity values were uninformative parameters in terms of survival.

3.2 Elasticities, population growth rates and extinction risks

Survival of the mature vegetative and fertile individuals had the highest elasticities (Tables 1 and A.7). Recruitment had an elasticity different from zero only in large populations (Tables 1 and A.7), and elasticities for transitions of dormant stages were low (0.000-0.088). The importance of recruitment on the population growth rate was higher in large populations (N > 100) with population growth rates that exceeded one compared to small populations (Fig. 4). In small populations, recruitment did not contribute to the population growth as these populations were on the F=0 vertex (Fig. 4).

The deterministic population growth rate varied among populations (Table 1) and was significantly lower in the small populations (λ_d = 0.993-0.994) compared to the large populations (λ_d = 1.000-1.016) ($F_{1,9}$ = 51.902, p < 0.001, Fig. 5) and a significant positive correlation between population growth rate and the population size was observed (R^2 = 0.779, p = 0.005). This correlation was observed also, when the analyses of the large populations were limited to the last three monitoring years that were common among the small and large populations ($F_{1,9}$ = 7.343, p = 0.024). The stochastic growth rate varied from 0.973 to 0.993 in the large populations (Table 3). The population extinction probabilities ranged from 0.036 to 0.934 within the next 100 years, and only one population was considered viable according to the used criteria (Fig. 6). The same trends in the elasticity values were observed from the stochastic matrix model as from the deterministic model (Table A.8, Fig A.1).

An increase in the initial population size decreased the probability of quasi-extinction. Quasi-extinction probability was 0.967, 0.663, 0.253 and 0.026 for an initial population sizes (N_0) of 50, 100, 200 and 500 individuals, respectively (Fig. 6).

3.3 Genetic analyses

An excess of homozygotes indicated null alleles in three populations for Ccal-25 (Patoköngäs, Kiutaköngäs N, Ampumavaara) and for SW2-185 (Kiutaköngäs N, Kiutaköngäs E, Kiutaköngäs SWc). Indications for scoring errors due to stutter bands were observed in three populations for SW2-185 (Kiutaköngäs N, Kiutaköngäs E, Kiutaköngäs SWc). Due to the absence of signs of null-alleles or indications of stutter bands in other populations, we used all the six loci in further analyses. The mean error rate was 0.12 per locus and 0.06 per allele. No linkage disequilibrium was detected between the loci. Deviations from the Hardy-Weinberg equilibrium were found in four populations (Table 4) as a deficit of heterozygotes, possibly indicating inbreeding. All loci had low levels of polymorphism with only two (SW1-76, SW2-152), three (Ccal25, SW2-185 and SW1-120), or five (SW1-78) alleles per locus. The genetic diversity estimates are given in Table 4. Allelic richness increased with population size, however this correlation was not significant (R^2 = 0.423, p = 0.223). Instead, allelic richness correlated with the deterministic population growth rate (R^2 = 0.680, p = 0.031). The heterozygosity was not linked to population size (R^2 = 0.052; p = 0.887) nor the deterministic population growth rate (R^2 = 0.201; p = 0.577). Individual heterozygosity ranged from 0 (homozygous over all loci) to 1 (heterozygous in all loci) and differed significantly between populations ($F_{9,186}$ = 2.231, p = 0.022) but was not significantly lower in the small populations compared to the large populations ($F_{1,194}$=1.191, p = 0.277). Young and old individuals did not differ

in their heterozygosity ($F_{1,192} = 0.026$, $p = 0.872$). The inbreeding coefficient (F_{IS}) was not correlated with the population size ($R^2 = 0.105$; $p = 0.772$).

4. Discussion

4.1 Viability

In this study, we incorporated both demographic and genetic parameters to assess their effects on the viability of an endangered perennial orchid, which enabled us to identify critical life history stages for small populations and the potential mechanisms limiting these stages. We showed that the viability of small populations ($N < 100$) was remarkably lower compared to large populations. Small populations had a significantly lower deterministic growth rate due to remarkably low recruitment rates. While the three year monitoring of the small populations was likely too short to capture the full demography, the recruitment over the three year period was extremely low, being zero in all of them, except in Kiutaköngäs SWc. This population was the largest among the monitored small populations, indicating that a certain minimum population size is required for successful recruitment.

4.2 Low seedling recruitment

Lowered seedling recruitment rates appeared to be the bottleneck for viability in small orchid populations. Low fecundity can be caused by both ecological and genetic processes. In small populations, ecological processes can decrease the reproductive output. These processes may include failure to attract pollinators or problems in plant-pollinator interactions, resulting in low seed production (Ågren 1996; Sih and Baltus 1987). Orchids are particularly vulnerable to pollen limitation due to their pollinator specialisation and decreased fruit set has indeed been observed in several small orchid populations (Abeli et al. 2013; Gijbels et al. 2015). However, we observed capsules also in the small populations, suggesting that flowers did not fail to set fruits due to pollinator deficit. Further, we did not find any correlation between fruit set and population size. Other studies have also revealed a lack of a correlation between the fruit set and the population growth rate in orchids and that other factors, such as seedling recruitment rate, were determinants of population viability (Calvo and Horvitz 1990; Calvo 1993).

Successful recruitment depends both on the availability of seeds and suitable microhabitats (Nathan and Muller-Landau 2000). In orchids, recruitment is limited by the availability of microhabitats rather than the fruitset (Kull 1998). Orchids are characterised by very strict habitat conditions, both biotic and abiotic. Successful seedling recruitment has been shown to be determined mainly by the presence of suitable mycorrhiza (Bidartondo et al. 2008), as orchids depend on mycorrhizal associations for germination of their small seeds that lack sufficient nutrients (Rasmussen 1995; Arditti and Ghani 2000). Successful seedling recruitment may be further restricted by the strong mycorrhizal fungal specialisation (Shefferson et al. 2007). This specialisation has been shown to be more pronounced during the protocorm stage in some species, including *E. atrorubens* (Bidartondo and Read 2008; Bidartondo et al. 2004; McCormick at al. 2004; Shefferson et al. 2008). High fungal specialisation can have strong implications for the conservation of orchids (McCormick et al. 2004), as the distribution of mycorrhiza determines the establishment of new individuals and thus the spatial distribution of orchids (Rasmussen 1995; Bidartondo et al. 2004). There are indications that the abundance of mycorrhiza decreases with increasing distance from adult plants (Batty et al. 2001; Diez 2007). Seedling recruitment is therefore expected to be higher near dense sites due to a higher abundance of fungi associated with the roots of the adult plants. The amount of suitable microhabitats can therefore be higher in large populations compared to small populations. This mechanism is effectively an Allee effect (Lamont et al. 1993; Courchamp et al. 1999). The small populations contained proportionally more fertile and thus older individuals compared to large

populations, indicating that small populations are likely remnants of once larger populations. Low quality or quantity of microhabitats suitable for successful germination might be behind the low recruitment rates in the small populations. However, based on our results no inferences on this can be made and more research is needed to test for the influence of microhabitat conditions, such as fungal abundance, on the germination rates.

4.3 Genetic variation

We found positive inbreeding coefficients in almost all populations, suggesting that inbreeding may occur. In small populations, higher probabilities to mate with related individuals and increased selfing rates through geitonogamy or autogamy due to pollinator deficit are expected. In the last case, pollination limitation cannot be observed as a reduced fruit set, as individuals do not fail to produce seeds.

Increased selfing and mating with relatives, might reduce the fitness through a lowered fecundity and decreased survival and growth rates, as a result of the expression of harmful and deleterious recessive alleles in homozygotes (Ellstrand and Elam 1993; Reed and Frankham 2003; Young et al. 1996). Many studies that have shown the negative effects of inbreeding on individual and population survival (Keller and Waller 2002; Reed and Frankham 2003) and reproductive success (Ferdy et al. 2001; Sletvold et al. 2012). However, despite of low observed genetic diversity (as compared to other orchids; e.g. Gustafsson 2000; Soliva and Widmer 2003) and significant inbreeding levels, we found no indications of inbreeding depression. No correlation between population size and genetic variation (allelic richness, heterozygosity or the inbreeding coefficient) was observed, suggesting that the low seedling recruitment observed in small populations is not due to genetic factors. These results contrasts with meta-analyses on other orchids and plants that show decreased genetic variation in small populations (Aguilar et al. 2008; Gijbels et al. 2015 Honnay and Jacquemyn 2007; Leimu et al. 2006). Further, the heterozygosity was not significantly different in young (non-fertile stage) versus old plants. Additionally, no indications for a lower survival due to inbreeding were found using the mark-recapture analyses. A similar lack of a relationship between the inbreeding coefficient and population size has been observed in several meta-analyses in orchids (Gijbels et al. 2015) and other plants (Aguilar et al. 2008; Honnay and Jacquemyn 2007; Leimu et al. 2006). The lack of a correlation in this study may be explained by historically low variation and purging of deleterious alleles in these populations or by the low number of markers and low levels of polymorphism in our study populations. Despite the lack of a correlation of the genetic variation with the populations size, the allelic richness correlated positively with the deterministic population growth rate. While the population size represents only a momentary point of the population, the population growth rate represents the long-term dynamics of populations. Allelic richness may be a better correlate for the long-term than for the short-term dynamics. These results are interesting as there is not much empirical evidence on the genetic effects on long-term population viability.

4.4 Temporal stochasticity

We observed high levels of inter-annual variation in the demographic parameters within populations. Variation was especially noticeable in the seedling recruitment rate that showed substantial temporal and between population variation. The observed variation affected the population viability by reducing population growth rates. The stochastic growth rates (λ_S) ranged from 0.973 to 0.993, indicating slight declines. This was also reflected by the probability of extinction (P_e), which suggested extinction of all large populations ($P_e > 0.050$) except for Ampumavaara ($P_e = 0.036$) during the next 100 years. Stochasticity may play an important role in the persistence of small populations, populations that are at equilibrium or slightly growing can be affected in the long-term (Lande 1988; Shaffer 1981). Thus, maintenance of large population sizes is needed to buffer the negative consequences of stochasticity.

The effect of stochasticity on viability varied across the large populations. Populations Ampumavaara and Kiutaköngäs N had almost identical stochastic growth rates (λ_s = 0.992-0.993) and relatively low probabilities of extinction (P_e = 0.036-0.084). However, population Patoköngäs had a high probability of extinction (P_e = 0.934) and a low stochastic growth rate (λ_s = 0.973), reflecting a higher effect of stochasticity on the viability of this population. This was due to the low seedling recruitment and survival rate in Patoköngäs. The seedling recruitment rate was lowest in Patoköngäs (F = 0.028) among the large populations (F = 0.051, Ampumavaara and F = 0.082, Kiutaköngäs N). In this population no seedling recruitment was observed in nine years. In addition, the average seeding survival rate of Patoköngäs (survival = 0.600) was the lowest among the large populations (survival was 0.889 in Ampumavaara and 0.889 in Kiutaköngäs N). This pattern was observed using both the survival values estimated with the matrix approach and multi-state mark-recapture method. Further, the variation of the seedling survival rate was high in this population.

When the large populations were combined to simulate the extinction probability using different initial population sizes (N0), the increase in population size enhanced population viability by buffering against the inevitable decline caused by stochasticity. An initial population size of >300 individuals was required for a viable population.

4.5 Conservation implications

E. atrorubens is reported to have declined in Finland due to succession and human caused threats, such as trampling, collecting and mining (Rassi et al. 2010) and local population extinctions have been observed in the study region. Management of the remaining habitat fragments is needed in order to prevent further decline of suitable habitats.

We found indications that small populations are not viable over a long period, due to low recruitment rates. The low observed seedling recruitment in small populations is possibly linked to the lack of suitable microhabitats for germination rather than ecological or genetic processes, such as inbreeding depression, pollen limitation or pollinator deficit. This can have profound consequences to the conservation of orchids.

We identified stasis as the most important transition for the population growth rate. This is in line with other perennial species in which survival and growth have the largest effect on the population growth (Silvertown et al. 1993; 1996). However, in the studied populations the reproduction was low and consequently the recruitment had low elasticity values (0-0.020). They were highest in the large populations (N>100) that reproduced. Elasticity values for fecundities have been shown to be positively correlated with the population growth rate (Oostermeijer et al. 1996). Population growth occurs only when recruitment is different from zero, and population growth rates will decline below unity when the fruit set and seedling recruitment are severely limited (Silvertown et al. 1996). The low recruitment rate will eventually lead to the extinction of small populations. Elasticity values should thus not be interpreted without taking into account the corresponding growth rates (Oostermeijer et al. 1996; Silvertown et al. 1996), when planning conservation and management of endangered species. As survival rates were not particularly low compared to other similar orchids (e.g. Kéry and Gredd 2004; Shefferson 2006; Shefferson Kali 2006; Shefferson et al. 2001;2003), our results strongly suggest that management should focus on enhancing recruitment. We note, however, that we were unable to conclude anything about mortality in small vs. large populations. Survival rates could be higher for example due to reduced density dependence effects from competition or lower due to unfavourable microhabitats.

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Tables

Table 1. Basic demographic parameters of the study populations obtained from the deterministic matrix models. Number of monitored individuals per population (N), the maximum counted individuals including those outside the monitored plot, time of monitoring in years (T), recruitment (R), population growth rate (λ_d), elasticity of the survival of seedlings (e_s), vegatatives (e_v), mature vegetatives (e_{mv}) and fertiles (e_f), and recruitment (e_r). Note that the elasticity's do not sum up to 1, as elasticity's of dormant stages are not shown.

Population	N	$N_{incl. outside plot}$	T	R	λ_d	e_s	e_v	e_{mv}	e_f	e_r
Ampumavaara	247	313	16	0.051	1.009	0.016	0.078	0.319	0.449	0.016
Kiutaköngäs N	171	171	16	0.082	1.016	0.019	0.114	0.383	0.320	0.019
Patoköngäs	152	315	14	0.028	1.006	0.008	0.036	0.237	0.549	0.008
Mataraniemi	134	>500	3	0.024	1.000	0.007	0.043	0.497	0.397	0.007
Kiutaköngäs SWc	65		6	0.020	0.994	0.000	<0.001	0.221	0.715	0
Kiutaköngäs b	32		3	0	0.993	0.000	<0.001	0.042	0.916	0
Kiutaköngäs E	36		3	0	0.993	0.000	<0.001	0.040	0.960	0
Kiutaköngäs NWb	30		3	0	0.993	0.000	0.000	1	0	0
Kiutaköngäs NWc	11		3	0	0.993	0.000	0.000	0.000	1	0
Kiutaköngäs SWa	15		3	0	0.994	0.000	0.000	0.310	0.595	0
Kiutaköngäs W	17		3	0	0.993	0.000	<0.001	0.336	0.613	0

912 Table 2. Survival rates of the large populations estimated with the matrix approach and multi-state
913 mark-recapture methods. Only three large populations were used for the mark-recapture analyses.

Stage	Matrix approach	mark-recapture	
		Estimate	CI
Ampumavaara			
Seedling	0.889	0.766	0.614-0.870
Vegetative	0.966	0.914	0.886-0.935
Mature vegetative	0.991	0.962	0.942-0.976
Fertile	0.988	0.972	0.955-0.982
Kiutaköngäs N			
Seedling	0.967	0.968	0.712-0.997
Vegetative	0.978	0.936	0.908-0.956
Mature vegetative	0.993	0.978	0.957-0.989
Fertile	0.992	0.970	0.943-0.985
Patoköngäs			
Seedling	0.600	0.429	0.189-0.708
Vegetative	0.973	0.967	0.933-0.984
Mature vegetative	0.995	0.990	0.943-0.998
Fertile	0.998	0.984	0.949-0.995

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Table 3. Basic demographic parameters based on the stochastic matrix analyses of populations Ampumavaara, Kiutaköngäs N and Patoköngäs. The number of individuals during year 2014 (N_{2014}), the number of individuals used a starting populations for the simulations (N_0), the stochastic growth rate (λ_s), the probability of extinction (P_e), the viable time (t_{viab}) and the elasticity of the survival of seedlings (e_s), vegatatives (e_v), mature vegetatives (e_{mv}) and fertiles (e_f), and recruitment (e_r). Note that the elasticity's do not sum up to 1, as elasticity's of dormant stages are not shown.

Population	N_{2012} N_{2014}	N_0	λ_s (C.I)	P_e (100years, 25 ind)	t_{viab}	e_s	e_v	e_{mv}	e_f	e_r
Ampumavaara	119	139	0.992 (±0.001)	0.036 (±0.003)	106	0.015	0.083	0.317	0.468	0.014
Kiutaköngäs N	133	133	0.993 (±0.001)	0.084 (±0.004)	85	0.014	0.082	0.414	0.346	0.014
Patoköngäs	86	145	0.973 (±0.001)	0.934 (±0.005)	36	0.002	0.030	0.217	0.631	0.002

951 Table 4. Genetic diversity estimates for each population. The sample size (N), expected
952 heterozygosity (He), observed heterozygosity (Ho), allelic richness (A_R), inbreeding coefficient (F_{IS})
953 and the p-value for Hardy-Weinberg equilibrium. The indicative adjusted nominal level (0.05) is
954 0.00083. He, Ho, AR and their standard deviation are calculated as the means over loci. No genetic
955 diversity estimates were calculated for population Kiutaköngäs NWc, as only one individual was
956 sampled for this population.

Population	N	H_o ±SD	H_e ±SD	F_{IS}	p-value	A_R±SD
Patoköngas	30	0.317±0.246	0.351±0.185	0.096	0.136	1.841±0.408
Kiutaköngäs N	30	0.289±0.154	0.439±0.128	0.347	**<0.001**	2.084±0.389
Ampumavaara	30	0.204±0.163	0.242±0.124	0.160	**0.040**	1.598±0.255
Mataraniemi	25	0.312±0.174	0.397±0.206	0.220	**0.014**	1.850±0.634
Kiutaköngäs SWc	22	0.203±0.067	0.300±0.166	0.331	**0.011**	1.478±0.433
Kiutaköngäs b	11	0.238±0.111	0.225±0.099	-0.059	0.694	1.524±0.353
Kiutaköngäs E	12	0.287±0.258	0.378±0.197	0.253	0.064	1.440±0.550
Kiutaköngäs NWb	9	0.342±0.061	0.361±0.102	0.059	0.386	1.747±0.393
Kiutaköngäs SWa	11	0.198±0.272	0.256±0.261	0.236	0.094	1.491±0.694
Kiutaköngäs W	16	0.439±0.187	0.505±0.135	0.137	0.142	1.845±0.714

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Figures

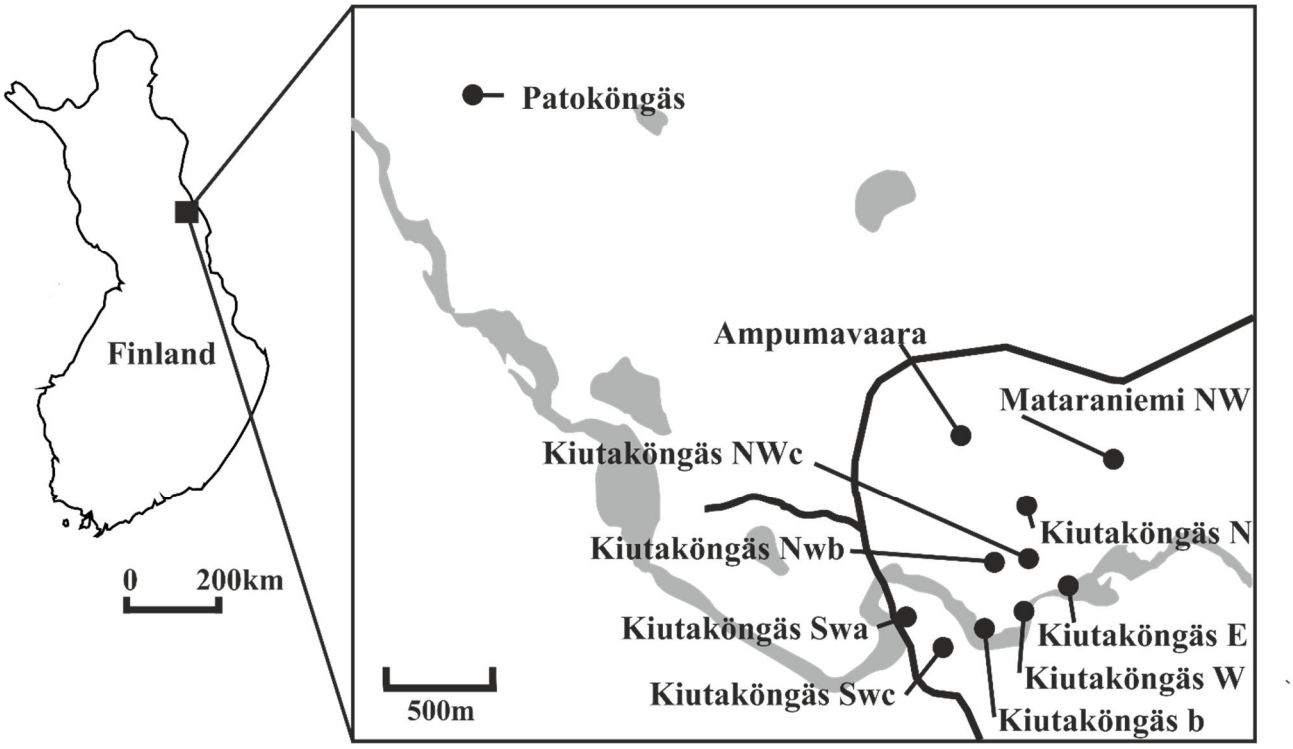

Fig. 1. Map of the studied populations. Black lines represent roads. Grey areas represent rivers or lakes.

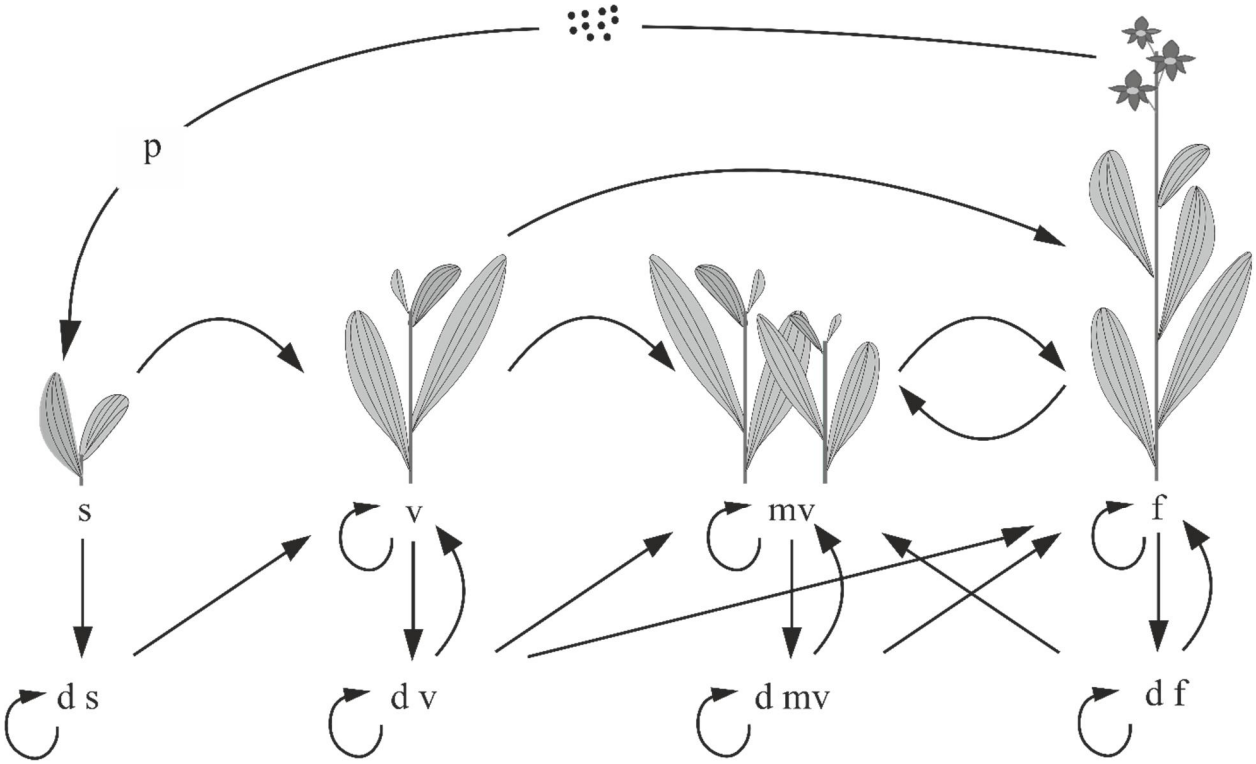

Fig. 2. Stage-based life cycle graph for *E. atrorubens*. Life stages are protocorm (p), seedling (s), vegetative (v), mature vegetative (mv), fertile (f), dormant seedling (ds), dormant vegetative (dv), dormant mature vegetative (dmv) and dormant fertile (df). Arrows represent possible transitions. Matrix elements (aij) corresponding to the transition matrix are indicated for growth (Gij), stasis (Sij), and reproductive (Fij) transition rates.

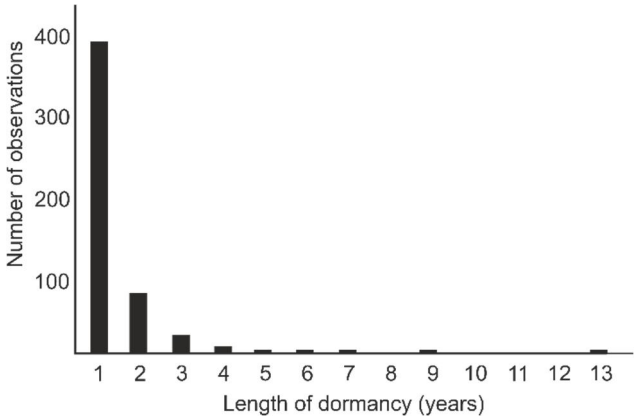

Fig. 3. Distribution of the length of dormancy including all life-history stages.

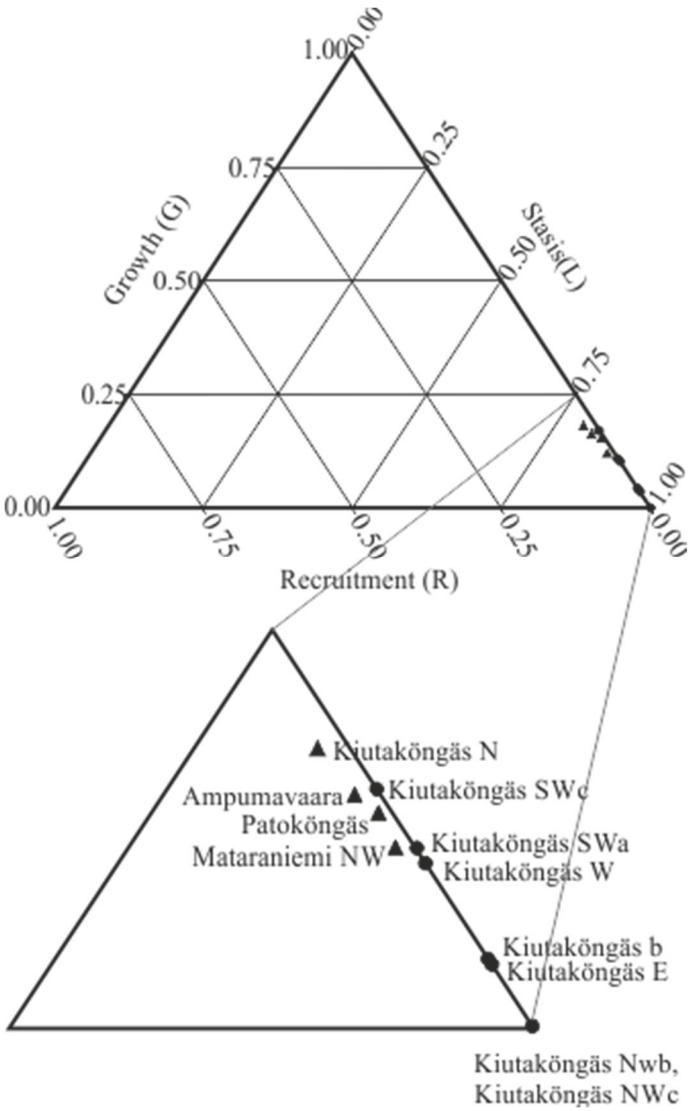

Fig. 4. Elasticities of growth (G), stasis or regression (L), and recruitment (R) for deterministic stage-based matrices of each population plotted on a ternary graph according to Silvertown et al. (1993). Small populations (N<100) are given as circles and large populations (N>100) are given as triangles.

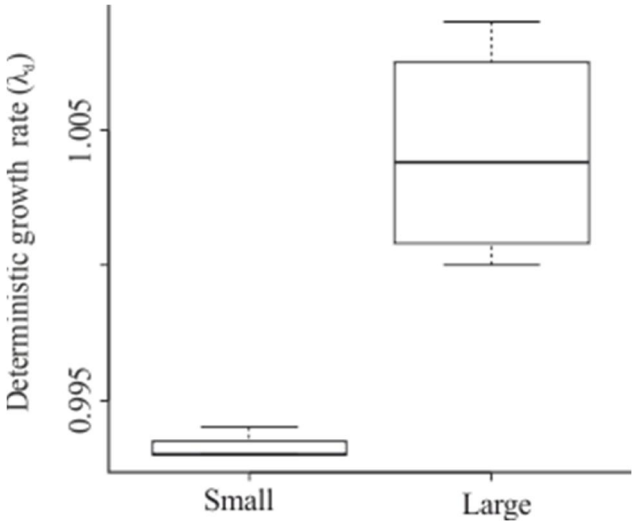

Fig. 5. Deterministic growth rate (λ_d) for small (N<100) and large populations (N>100).

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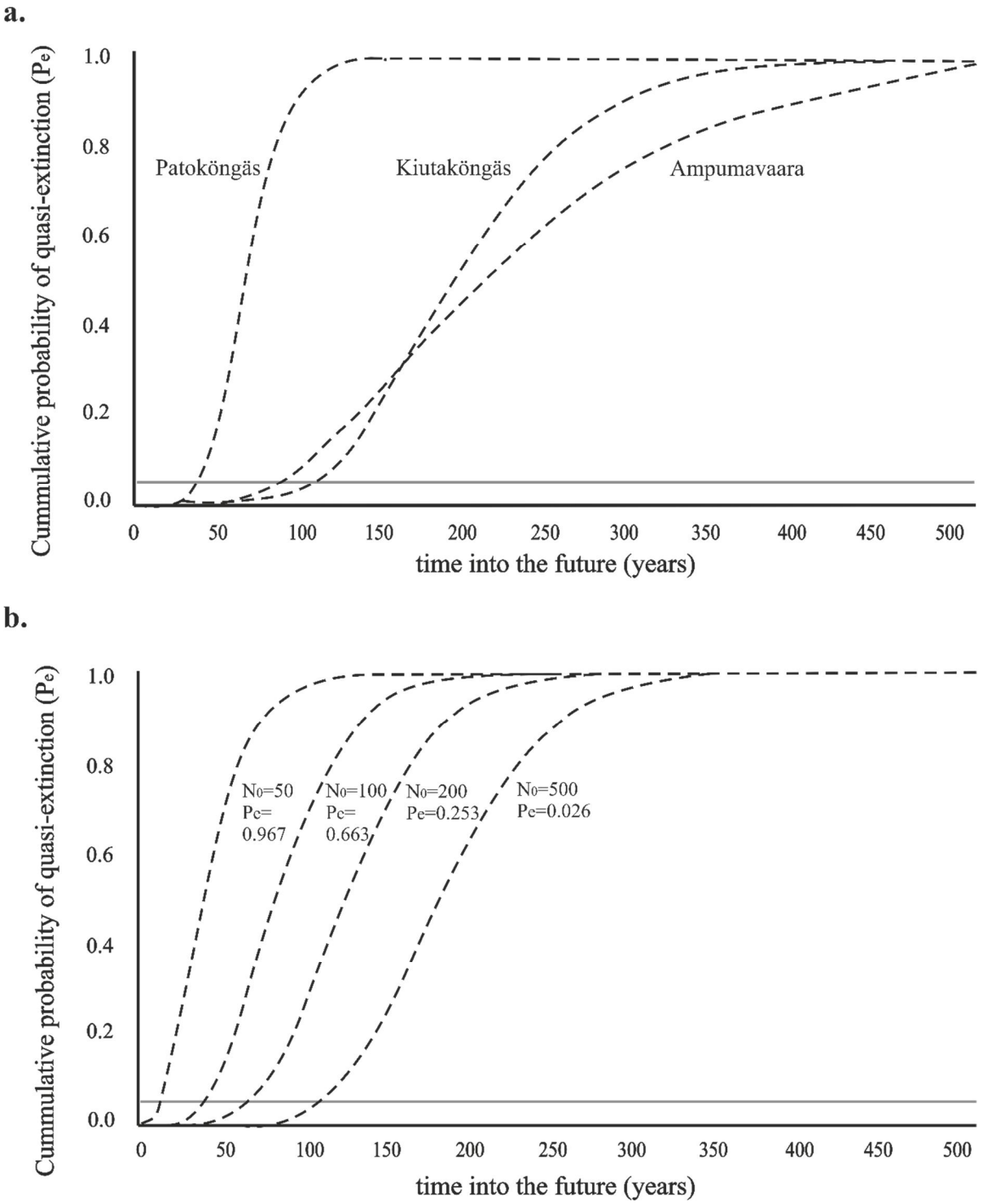

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1092 Fig. 6. (a) Probability of extinction (P_e) for populations Patoköngäs, Kiutaköngas N and
1093 Ampumavaara. (b) Probability of extinction (P_e) for different hypothetical starting population sizes
1094 (N_0), for the large populations combined. The projections were carried out for 500 years. The quasi
1095 extinction threshold was 25 individuals. The grey line indicates the 5% viability threshold.