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Cryptic genetic bottlenecks during restoration of an endangered tropical conifer

Chris J. Kettle[a,b,*], *Richard A. Ennos*[b], *Tanguy Jaffré*[c], *Martin Gardner*[a], *Peter M. Hollingsworth*[a]

[a]*Royal Botanic Gardens Edinburgh, 20a Inverleith Row, Edinburgh EH3 5LR, UK*
[b]*Institute of Evolutionary Biology, School of Biological Sciences, Ashworth Laboratories, University of Edinburgh, Edinburgh EH9 3JT, UK*
[c]*IRD Institut de la recherche pour le développement, Centre de Nouméa, Laboratoire de Botanique et d'Ecologie Végétale Appliquées, BPA5 – 98848 Nouméa, New Caledonia*

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ABSTRACT

Forest restoration programmes aim to use material for re-planting that is genetically diverse and not inbred. However, restricted seed sampling, high variance in reproductive output, and the production of inbred seeds that survive in the nursery but not in the wild can lead to forest restoration stock being genetically compromised. The aim of this study was to evaluate whether the reproductive biology of the New Caledonian endemic conifer *Araucaria nemorosa* makes it susceptible to these genetic problems and to assess whether there is evidence for genetic bottlenecks and elevated inbreeding in nursery stock compared to seedlings and adults from wild source populations. Reproductive output was low with high variance among trees (only 14% of adult trees surveyed produced mature cones, >50% of examined cones had <10 viable seeds). Evidence for an extreme genetic bottleneck was detected in a nursery population established from cones collected from adult trees. A second nursery population established with seed collected from the forest floor showed no evidence of a genetic bottleneck, but was inbred compared to its wild source population. In light of these results, we do not recommend collecting cones directly from *A. nemorosa* as an efficient means of establishing genetically diverse stock for restoration programmes. Collecting seed from the forest floor is likely to be more effective, but the planting stock may contain a high proportion of inbred individuals. Collecting established wild seedlings already subjected to natural selection is suggested as an alternative method of maximising the diversity captured, whilst minimising sampling effort and proportion of inbred individuals.

1. Introduction

Forest restoration is becoming increasingly important given the current wide-scale degradation and fragmentation of forest habitats. Over the short term forest restoration programmes are likely to be most successful if re-planting stock is of high immediate fitness, free from the deleterious effects of inbreeding (Charlesworth and Charlesworth, 1987) and locally adapted to the current conditions at the site of restoration (Hufford and Mazer, 2003; McKay et al., 2005; O'Brien et al., 2007). The long-term success of forest restoration programmes will be improved if the re-planting stock

* *Corresponding author:* Present Address: Ecosystem Management, Institute of Terrestrial Ecosystems, ETH Zurich, CHN G73.2 Universitaestrasse 16, Ch-8092 Zurich, Switzerland. Tel.: +41 0 44 632 31 95; fax: +41 0 44 632 15 75.
E-mail addresses: Chris.kettle@env.ethz.ch (C.J. Kettle), rennos@ed.ac.uk (R.A. Ennos), Tanguy.Jaffre@noumea.ird.nc (T. Jaffré), M.Gardner@rbge.ac.uk (M. Gardner), P.Hollingsworth@rbge.ac.uk (P.M. Hollingsworth).
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possesses the adaptive potential to respond to future environmental change. Forest restoration efforts should thus aim to use material for re-planting which avoids inbred progeny and maximises the retention of genetic diversity, while ensuring that individuals are suitably adapted to the local site conditions. There are a number of factors associated with seed collection practices and the reproductive biology of trees, however, which may lead to the genetic quality of forest restoration material being compromised.

Problems may arise firstly as a consequence of seed collection procedures. If seed is harvested from a restricted sample of the source population, its genetic base may be very narrow. This may arise where collections are spatially restricted and seed is collected from very few individuals or from trees distributed over a very limited range of ecological conditions within a site. This problem is illustrated by a study of 148 seed lots raised in East African tree nurseries. Lengkeek et al. (2005) estimated that, on average, only 6.4 maternal parents contributed genetic material to any one of these seed lots. Other examples of restricted sampling leading to a narrow genetic base in forest restoration material have been documented in conservation programmes based in Scotland and Sicily (Ennos, 2003; Burgarella et al., 2007). A factor that adds further restrictions to the sampling of adult gene pools is that collections are typically made in only one, or at most, a very few years. In a given season, not all adults in a population will be reproductively active. This may limit the genetic diversity, present in the adult population, that can be captured in the seed sample.

A second factor that may lead to reduced genetic diversity in the seed collections used to generate nursery stock, is high variance in fertility between reproductively active individuals. A few individuals may contribute disproportionately to the next generation resulting in a "cryptic" genetic bottleneck (Luikart et al., 1998). Variance in fertility can contribute to the rapid accumulation of relatedness and inbreeding in subsequent generations. Large differences in fertility among individual trees have been reported in a number of conifer species (Savolainen et al., 1993; Bilir et al., 2003) and this has been of concern in maintaining the genetic base of seed collected from seed orchards and seed stands of commercial forestry species (Muona and Harju, 1989; Kang and Lindgren, 1998; Bilir et al., 2003). For example, it has been estimated that only 20% of clones produce 80% of the seed in most clonal conifer seed orchards (El-Kassaby, 1995). Furthermore, in species that bear variable proportions of morphologically indistinguishable viable and non-viable seeds, variance in fertility may not be recognised as a problem by seed collectors who rely on seed number to assess the contribution of adults. In such situations even a carefully designed sampling strategy can result in a nursery stock dominated by the progeny of a small number of parents.

A third factor that may compromise the genetic quality of seed used for forest restoration is an increase in inbreeding during the transition from adult to seed generations. It is well documented that many self-compatible hermaphrodite trees show a mixed mating system with progeny derived from both self-fertilisation and outcrossing. Examination of the inbreeding coefficients of adults and seedlings often shows an increase in heterozygosity with cohort age (Yazdani et al., 1985; Strauss and Libby, 1987). Selection against inbred individuals in the wild leads to preferential survival of trees derived from outcrossed matings. Over the course of a complete generation the lower inbreeding levels present in adults are ultimately re-established in their offspring. Thus a seed sample collected for restoration work may contain a mixture of outcrossed and selfed individuals, of which the selfed individuals would not naturally survive in the wild. If nursery conditions are benign compared to the wild, the expectation is that there will be greater survival of these inbred individuals than expected under natural conditions. This leads to a prediction of lower growth rates and high mortality if this material is subsequently used as stock for restoration.

With the above issues in mind, it is clear that assessing whether the material used for restoration programmes is genetically compromised is not straightforward. Some insights into whether there are likely to be significant genetic problems in the nursery material can be gained by conducting a simple baseline survey of the reproductive behaviour of the relevant populations. Measures of the variability in flowering behaviour and viable seed set among individuals will indicate whether the conditions exist that might lead to high variance in contribution of adults to the next generation, and a cryptic bottleneck.

A complementary approach for retrospectively assessing the efficiency of restoration programmes in capturing the genetic variation of wild populations is to measure the levels of neutral genetic diversity at molecular markers in the nursery material and compare this with the source populations. If nursery material shows a significant reduction in genetic diversity this may indicate either a poor sampling strategy or some other problems with the seed collection, such as a "cryptic" genetic bottleneck. At the same time inbreeding coefficients can be assessed in nursery and adult material to determine whether the potential problems associated with production of inbred planting stock are likely to be significant.

A further refinement of this approach is to include samples of naturally established seedlings in the study. By comparing genetic diversity and inbreeding in naturally occurring seedlings and nursery material it should be possible to disentangle genetic changes that occur naturally between adult and seedling cohorts in the wild, from additional genetic changes associated with the nursery seed collection regime.

In this paper, we report a baseline survey of reproductive behaviour in populations of a critically endangered New Caledonian conifer. We then compare genetic diversity and structure among adults, naturally occurring seedlings, and two nursery stocks of this species. Our objective is to assess the extent to which the genetic quality of the nursery material is compromised, and identify any natural or management related processes that may be involved.

2. Materials and methods

2.1. *Study system*

The Pacific Island archipelago of New Caledonia (Fig. 1) is a global biodiversity hotspot (Myers et al., 2000). It is the sole

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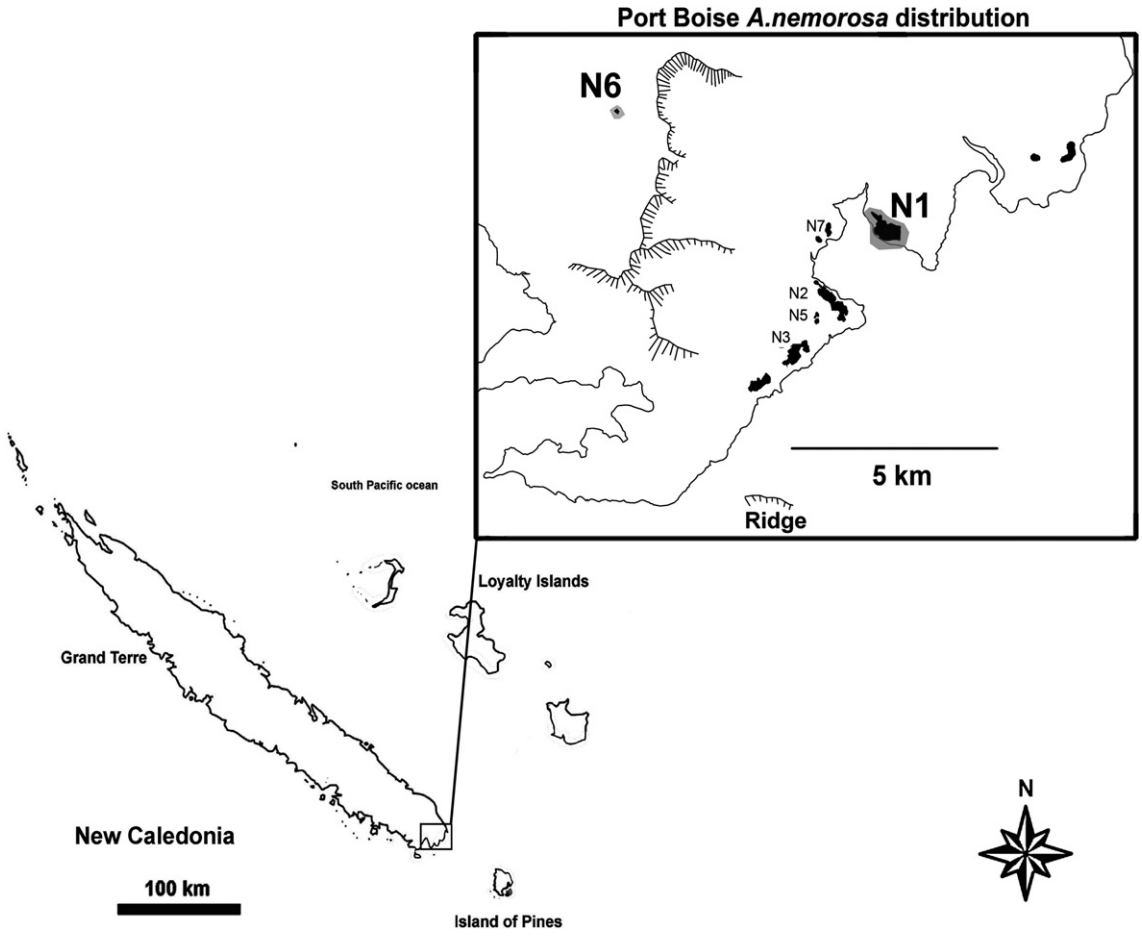

Fig. 1 – Map of New Caledonia showing the distribution of *Araucaria nemorosa*. The insert box shows the location of all remnant populations (black polygons). Populations used only for the reproductive biology survey are indicated in small text. The two population used in both the reproductive biology survey and for collection of nursery material, Kaanua (N1) and Foret Nord (N6), are indicated in the large text and outlined in grey.

location of 13 of the world's 19 *Araucaria* species, 11 of which are included in the Global Red List of Conifers (Watt, 1999). Increased fire frequency, introduction of mammals, logging and mineral mining have resulted in extensive habitat degradation and less than 30% of the original vegetation on New Caledonia remains. Much of this habitat disturbance has occurred in the last 150 years (Jaffre et al., 1998).

The rarest of the iconic New Caledonian *Araucaria* is the critically endangered *Araucaria nemorosa* de Laubenfels. This long-lived hermaphrodite tree species is extant in only eight known populations, with an area of occurrence of 9.8 km^2 and an area of occupancy of 0.64 km^2 (Kettle, 2006). *A. nemorosa* regenerates sexually by producing large female cones each of which bear several hundred seeds. However many of the seeds produced, though apparently normal in appearance, are inviable (McCoy personal communication, 2003), restricting the regenerative potential of the populations. Furthermore, a recent study has demonstrated emerging genetic problems in wild seedling cohorts of *A. nemorosa* including a reduction in genetic diversity and elevated inbreeding (Kettle et al., 2007). This evidence demonstrates a clear need to develop restoration strategies for *A. nemorosa* that will augment current population numbers, re-establish populations on former sites and prevent escalation of the genetic problems. To achieve these restoration objectives, seedlings are currently being reared in the nursery for planting on open ground that has been denuded of vegetation following mining activity or fire disturbance.

Two contrasting methods have been used to obtain *A. nemorosa* seed for these restoration programmes. The first is directly to collect cones from mature trees. The second involves gathering freshly fallen seed from the forest floor. Prompt collection of fallen seed is necessary because seed germination takes place within a number of days of release from the cone. In this study we compare the genetic attributes of two nursery stocks of *A. nemorosa*, one derived from a cone collection and the other from seed gathered from the forest floor.

2.2. General assessment of cone production in *A. nemorosa*

To provide baseline information on reproductive biology of *A. nemorosa,* we undertook a survey to estimate the density of adults of reproductive size, the proportion of these trees bearing cones, and the variance in female cone production be-

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Table 1 – Distribution of second year cone production by A. nemorosa within two 0.04 ha plots in each of six natural populations

Population	Code	Area (ha)	N	Total sample	Trees >15 cm dbh	Number of trees with cones	Number of cones per coning tree	Mean dbh of trees (cm)	Minimum size (cm dbh) of cone bearing tree
Kaanua	N1	22.68	>1000	25	13	1	2	14.9	16
Vane	N2	15.72	>1000	15	8	1	7	25.6	48
New Forest	N3	15.2	>1000	63	45	4	1, 2, 3, 3	18.2	18.5
Mini Nuri	N5	1.28	<500	36	22	4	2, 2, 7, 5	18.4	22.5
Foret Nord	N6	0.44	93	28	11	5	1, 1, 1, 3, 9	17.1	32.7
Natasha's	N7	1.48	<100	62	23	2	2, 8	12.9	23.5

N is the estimated size of the population based on counts of mature trees from photographs taken at high elevation or direct counts. dbh is diameter at breast height (1.4 m) or equivalent (see text).

tween reproductive age individuals. From December 2002 to February 2003, two randomly located 20 m × 20 m (0.04 ha) plots were established in each of six populations of *A. nemorosa* encompassing most of its known sites (referred to as N1, N2, N3, N5, N6, N7; Fig. 1 and Table 1). Within each plot all *A. nemorosa* trees with a stem diameter >2 cm were recorded. To establish size distribution, diameter at breast height (dbh) was measured in individuals above 1.4 m tall. In the remaining smaller individuals, diameter was measured at a representative height on the stem. Each tree was scored for the presence and number of mature (second year) female cones. A total of 229 trees were included in this survey across all sites.

To make a preliminary assessment of the number and variability of viable seed set per cone, a small number (13) of second year cones were collected in February 2003 from populations N1 (1), N3 (4), N5 (6) and N7 (2). Cone collections from Foret Nord (N6) could not be conducted at the time of cone ripening due to access restrictions as a consequence of mining activities. Cones were dissected and the total number of seeds per cone was recorded. To investigate the number of viable seed per cone, each seed had its wings removed and was placed in a basin of water for five minutes. The proportion of seeds which floated was used to obtain a minimum estimate of non-viable seeds, because sterile seeds lacking embryos invariably float. Seeds which sank were assumed to be fertile. The mean and variance of (putatively) viable seed set per cone were calculated for populations N3, N5 and over all populations.

2.3. Genetic marker-based comparisons of nursery populations and their donor populations

The genetic marker studies focused on comparing the genetic diversity and level of inbreeding in two wild source populations with genetic diversity and level of inbreeding in the nursery populations derived from them. The two source populations in question are 'Kaanua' (N1) and 'Foret Nord' (N6) which have previously been analysed during a study on the genetic consequences of habitat degradation in *A. nemorosa* (Kettle et al., 2007). Kaanua (N1) is one of the largest populations of *A. nemorosa*. In contrast Foret Nord (N6) is the smallest, most isolated, and only inland population of *A. nemorosa*, located in the centre of a currently expanding nickel processing development (Fig. 1, Table 1).

In 2001, two nurseries made seed collections from these two populations for use in habitat restoration programmes and to investigate the silvicultural potential of *A. nemorosa*. The first nursery obtained a seed sample from Kaanua (N1), by collecting cones from approximately 30 adult trees. The second nursery sampled newly fallen seed from the forest floor of Foret Nord (N6). The area of forest floor over which collections were made is unknown. In both cases the seed was germinated in June 2001 and protected from fungal pathogens and seed predators. After germination, seedlings were potted out and reared under shade conditions. Germination rates were generally low (c. 30–40%; McCoy personal communication, 2003).

In order to assess the genetic diversity and inbreeding level of seedlings from these two nursery populations, 40 seedlings were sampled from each in February 2003 (see Table 3). The genetic diversity and inbreeding levels in the nursery populations were compared to those of wild established seedlings and adult trees sampled from the source populations N1 and N6 (Kettle et al., 2007). The sampling strategy for the adults and wild seedlings from the source population has been previously described by Kettle et al. (2007). Briefly, leaf needle material, derived from 40 paired (proximal) adult and seedlings from populations N1 and N6, was placed into silica gel (Table 1, Fig. 1). A distance of at least 5 m was maintained between each sample pair. Sampling extended over the entire range of each population. Wild seedlings (still with cotyledons) were estimated to range in age from between 1 and 5 years.

It is important to stress that the sampling of material for establishing the nursery stocks was carried out in 2001, whereas the sampling for genetic marker analysis and assessments of reproductive output was carried out in 2003. Thus, the information on reproductive demography cannot be used to directly account for any diversity and inbreeding differences in nursery stock compared to wild source populations. Instead, the reproductive biology survey is intended as a general indication of seed set and viability in the species.

2.4. DNA extraction and genotyping

DNA isolation and nuclear microsatellite (*n*SSR) analysis followed protocols described by Kettle et al. (2007). Briefly, total DNA was extracted from approximately 0.03 g of silica-dried needle material using the QIAGEN Mixer Mill and the QIAGEN

DNeasy 96-Well-Plate plant kits. All samples were screened for variation at seven unlinked *nSSR* loci using primer pairs isolated from New Caledonian *Araucaria*. Details of the primer and PCR conditions are given in Robertson et al. (2004) and Kettle et al. (2007). The PCR products were analysed using a Beckman Coulter CEQ 8800 genetic analysis system.

2.5. Data analysis

2.5.1. Genetic diversity, inbreeding coefficients, changes in rare allele frequency and genetic differentiation

The genetic marker data were used to assess whether there was evidence for (a) loss of diversity, (b) increase in inbreeding, (c) changes in rare allele frequency, and (d) genetic differentiation, in each of the nursery populations compared to their corresponding adult and seedling populations. The basic descriptive statistics allelic richness (A_E), gene diversity (H_e) and intra-population inbreeding coefficient (F_{IS}) were estimated for each of the three samples from each site using GDA (Lewis and Zaykin, 2001) and FSTAT version 2.9.3.2 (Goudet, 1995). As data analyses involved comparing variation at the same microsatellite loci in different samples (adults, wild seedlings, nursery seedlings) a paired *t*-test was used to determine the significance of differences in genetic diversity (A_E, H_e) using loci as replicates.

One of the principle genetic signatures resulting from population bottlenecks is loss of rare alleles (Cornuet and Luikart, 1996; Luikart et al., 1998). To test for preferential loss of rare alleles in the nursery samples the proportions of rare alleles (frequency <0.1) in each of the adult and wild seedling cohorts and nursery stock from a single site were calculated. A chi-squared test was used to determine whether there were significant differences in the proportions of rare alleles among these samples. This analysis provides a simple but powerful test for bottlenecks which does not depend on assumptions of random mating or Hardy-Weinberg equilibrium in sample populations.

To determine whether significant genetic differentiation exists between wild adults, and the wild seedlings and nursery seedlings derived from them, the pairwise F_{ST} values between these samples at each of the two sites were calculated, and their significance tested using the programme FSTAT version 2.9.3.3 (Goudet, 1995).

To obtain a quantitative estimate of the effective size of the population (N_e) giving rise to the nursery material, we estimated N_e from the change in gene diversity between generations using the relationship $N_e = H_0/[2(H_0 - H_1)]$ where H_1 and H_0 are gene diversities in nursery stock and adult source populations, respectively (Wright, 1931; Frankham, 2002). This method is only applicable in situations were there is a decline in gene diversity between wild source material and the nursery stock, otherwise the estimate of N_e is negative.

3. Results

3.1. Fertility variance and seed set in A. nemorosa

Cone production in all six populations of *A. nemorosa* sampled was very low with a high proportion of adult trees bearing no mature second year female cones (Table 1). The smallest tree to bear female cones was 16 cm dbh. Based on the assumption that all trees greater than 15 cm dbh were sexually mature ($n = 122$), the mean percentage of sexually mature trees bearing second year female cones was 13.9%, with 7.7% and 45.5% of mature trees bearing second year cones in Kaanua (N1) and Foret Nord (N6), respectively. Within populations there was evidence of a high variance in female cone production among individuals. The variance in cone production was twice and four times as great as the mean value in populations N5 (mean = 3; $\sigma^2 = 6$) and N6 (mean = 3; $\sigma^2 = 12$), respectively (Table 1).

Our preliminary assessment of seed set per cone, suggests that viable seed number can be very low in *A. nemorosa* with a high variance among cones (Table 2). The number of seeds (filled and unfilled) produced per cone averaged 339 and varied from 279 to 422. However, the percentage of filled seeds per cone was very low and highly variable. Over 50% of the cones produced less than 10 filled seeds, while a single cone produced 125 filled seeds. Thus over 50% of the putatively viable seed recovered from the collection of 13 cones was from a single cone.

3.2. Genetic diversity in adult, wild seedling and nursery seedling populations

All seven nSSR loci were polymorphic in wild seedling and adult cohorts from Foret Nord and Kaanua, and in the nursery sample from Foret Nord. There was no significant difference in allelic richness or gene diversity among wild seedling and adult cohorts in the samples from Kaanua ($P > 0.05$). However, the nursery seedling stock from Kaanua was monomorphic at locus As190 and had significantly lower allelic richness and gene diversity than the corresponding wild seedling and adult

Table 2 – Total seed production, number of filled seeds and % filled seeds per cone for each of 13 cones of A. nemorosa collected from four natural populations

Population code	Total seeds/cone	Filled seeds/cone	% Filled seeds
N1	422	13	3.08
N3	357	125	35.01
N3	395	15	3.8
N3	292	24	8.22
N3	279	12	4.3
N3 mean	**331**	**44**	**12.8**
N3 variance	**2998.9**	**2942**	**222.5**
N5	284	5	1.76
N5	282	2	0.71
N5	345	1	0.29
N5	313	4	1.28
N5	315	2	0.63
N5	306	12	3.92
N5 mean	**307.5**	**4.3**	**1.4**
N5 variance	**539.5**	**16.3**	**1.8**
N7	410	0	0
N7	405	1	0.25
Overall mean	**339**	**17**	**5**
variance	**2850.5**	**1112.1**	**87.4**

Table 3 – Genetic diversity measures, percentage of rare alleles (frequency <0.1) and inbreeding coefficients for adult (A), wild seedling (W) and nursery seedling (N) samples derived from two natural populations of A. nemorosa

Population	Code	Sample	Sample size	A_E	S.E(A_E)	H_e	S.E(H_e)	% Rare alleles	F_{IS}	N_e
Kaanua	N1	A	40	9.53	2.461	0.72	0.085	84	0.135*	
Kaanua	N1	W	40	8.83	1.901	0.71	0.088	74	0.194*	30
Kaanua	N1	N	40	2.81	0.463	0.46	0.093	38	–0.024n.s.	1
Foret Nord	N6	A	40	6.12	1.109	0.65	0.070	68	0.009n.s.	
Foret Nord	N6	W	40	5.59	0.901	0.62	0.073	65	0.168*	11
Foret Nord	N6	N	40	6.40	0.871	0.69	0.054	65	0.282*	*N/C*

A_E allelic richness, H_e gene diversity, F_{IS} inbreeding coefficient within samples. Significance of differences from $F_{IS} = 0$ are shown; *$P < 0.05$, n.s. non-significant. N_e effective size of populations giving rise to wild seedlings and nursery seedlings. *N/C* Not calculable (negative estimate obtained).

cohorts ($A_E = 2.8$ nursery seedling vs $A_E = 8.8$ wild seedling and $A_E = 9.5$ wild adult ($P < 0.05$); $H_e = 0.46$ nursery seedling vs. $H_e = 0.71$ wild seedling and $H_e = 0.72$ wild adult ($P < 0.05$)) (Table 3). In contrast, there was no significant difference ($P > 0.05$) in allelic richness or gene diversity between nursery seedlings from Foret Nord and either wild seedlings or adults from the same source population (Table 3).

3.3. *Proportion of rare alleles*

At Kaanua, the proportion of all alleles classified as rare (frequency <0.1) was much lower in the nursery seedling stock than in the wild seedling and adult cohorts ($\chi^2_{(2)} = 21.9$, $P < 0.001$). The nursery seedlings contain only 38% of alleles with a frequency of less than 0.1, while 74% and 84% of the alleles in wild seedling and adult cohorts, respectively, had a frequency below 0.1. In contrast there was no evidence for a difference in the proportion of alleles with a frequency of less than 0.1 among nursery seedlings, wild seedlings and adults at Foret Nord ($\chi^2_{(2)} = 0.132$, $P > 0.05$).

3.4. *Genetic differentiation*

Estimates of pairwise F_{ST}, measuring genetic differentiation between wild and nursery samples taken from the same site, indicated significant differentiation ($P < 0.05$) between nursery seedling stock and both adult and wild seedling samples at Kaanua ($F_{ST} = 0.160$ and 0.167, respectively). Significant genetic differentiation was also found between nursery seedling stock and adult and wild seedling samples at Foret Nord ($P < 0.05$), but the degree of differentiation was only a quarter of that found at Kaanua ($F_{ST} = 0.042$ and 0.047, respectively). No significant differentiation was detected between adult and wild seedlings cohorts at either site.

3.5. *Inbreeding coefficients*

There was no significant deficit of heterozygosity compared to Hardy-Weinberg expectations in the nursery seedling stock from Kaanua. Mean F_{IS} was −0.024 which did not differ significantly from zero. This contrasts with samples of wild seedlings and adults from the same site which exhibited significant F_{IS} values of 0.194 and 0.135, respectively (Table 3). At Foret Nord the situation was different. The inbreeding coefficient increased from adult ($F_{IS} = 0.009$) to wild seedling ($F_{IS} = 0.168$) to nursery seedling stock ($F_{IS} = 0.282$).

3.6. *Effective size of parent population*

The genetically effective size of the adult population contributing genes to the wild and nursery seedling cohorts was calculated based on the change in gene diversity between the adult and seedling samples. At Kaanua the estimated effective number of individuals contributing to the wild seedling cohort was large ($N_e = 30$), while the effective number contributing to the nursery stock was very small ($N_e = 1$) (Table 3). At Foret Nord, the estimated effective parent population contributing to the wild seedling cohort was moderate ($N_e = 11$). However, the observation of a slight increase in gene diversity in the nursery seedling stock compared with adults provides no evidence of a restricted population of adults contributing to nursery seedlings at Foret Nord (Table 3).

4. Discussion

4.1. *Fertility variance and seed set in A. nemorosa*

Female cone production and seed set are both very low in *A. nemorosa* and also have a high variance between individuals. In the current study, only a small proportion of potentially sexually mature trees produced mature cones. It is clear that seed collections made in any one year may be derived from only a small proportion of the adult population.

It has been reported that 10–40% of seeds are usually empty in conifer cones (Sorensen and Webber, 1997). Our preliminary investigation in *A. nemorosa* suggested even higher values with an average of 95% seeds empty per cone. Of nearly 4500 seeds collected, 219 were viable and 125 of these seeds were from a single cone. One tree contributed over 50% of the total seed collection from our sample. This demonstrates dramatically how a cryptic genetic bottleneck can occur due to high variance in fertility. This low level of seed set is similar to that recorded over 4 years from the rare Wollemi pine (*Wollemia nobilis*, Araucariaceae) which on average had less than 11% viable seeds per cone (Offord et al., 1999). Offord et al. (1999) suggest pollen limitation may be a cause of the low seed set in *W. nobilis*. An alternative explanation is embryo abortion due to inbreeding depression (Williams and Savolainen, 1996). Severe inbreeding depression at the embryo stage is thought to be common in conifer species (Muona and Harju, 1989; Hedrick, 2005). Evidence from controlled pollination experiments suggests that expression of deleterious recessives due to selfing is responsible for 80–96% of empty

seeds in conifers (Fowler and Park, 1983). The availability of viable outcrossed pollen may be reduced where there is increased spatial isolation between fertile individuals, and degradation and exploitation of tree populations can thus lead to increased levels of self-fertilisation (Murawski et al., 1994; Zheng and Ennos, 1999).

An additional potential source of low seed set that has been observed in other conifers is pollination by a congener resulting in empty seeds (Sarvas, 1962), presumably by swamping the reproductive structures with hetero-specific pollen. This is of potential relevance for *A. nemorosa* whose remnant populations are sympatric with very dense populations of the more widespread *A. columnaris,* which are likely to contribute significantly to the pollen cloud.

One qualification to our observation of limited seed set in *A. nemorosa* is that the data are based on observations from a single season. There may be considerable variation in seed set from year-to-year. Studies from another New Caledonian *Araucaria* (*A. laubenfelsii*) have identified the importance of mast years (Rigg, 1999). Having said this, the low level of coning in *A. nemorosa* has been apparent for a number of years (McCoy personal communication, 2003). Furthermore, in the context of relevance for restoration programmes, even if masting years occur, there is rarely the luxury of waiting for a year of exceptional seed production for seed collection. Instead, collections are undertaken when budgets are available for field work and growing space.

In summary, our reproductive ecology survey suggests that a small proportion of the adult population may contribute to seed production in any one year and that this may affect the diversity captured during sampling for nursery collections.

4.2. *Genetic marker assessments of the diversity in nursery seedlings compared to wild populations*

The results from this study indicate that nursery seedlings of *A. nemorosa* from Kaanua (N1) are more genetically depauperate than their wild relatives despite being established from seed collected from the largest and one of the most genetically variable remnant adult populations (Kettle et al., 2007). Thus allelic richness has dramatically declined in this nursery lot with the loss of many rare alleles and an associated very low estimate of N_e. In fact these nursery seedlings have captured less than 30% of the allelic diversity present in the source population and one locus (As190) is monomorphic. In contrast the nursery lot from Foret Nord has more effectively captured the available variation from its source population. Even though the Foret Nord source population is the most genetically depauperate of the remnant *A. nemorosa* populations (Kettle et al., 2007) the nursery stock has more than twice the allelic richness of the seed lot from Kaanua (N1).

An earlier study demonstrated that wild adult and seedling populations of *A. nemorosa* exhibit some level of inbreeding as indicated by significant positive inbreeding coefficients with the seedlings significantly more inbred than the adults (Kettle et al., 2007). Nursery seedlings from Kaanua however, did not show significant inbreeding coefficients but if anything slight heterozygous excess at most loci. One possible explanation for this somewhat paradoxical result is that the seedlings are the offspring of very few outcrossed parents (e.g. the result is representative of a small number of events, rather than an equilibrium-based measure of the population as a whole). This hypothesis is consistent with these nursery seedlings being derived from a very small number of individuals. In contrast, the nursery lot from Foret Nord has a significant positive inbreeding coefficient, which is considerably greater than the wild seedlings and the adults in the source population. In fact this inbreeding coefficient is higher than any we have recorded in *A. nemorosa* populations (Kettle et al., 2007). One explanation for this is that inbred progeny are under greater selection in natural forest conditions than in the comparatively benign and homogenous conditions of the nursery (some individuals survive in the nursery that would die in the wild). Thus, although wild seedlings have a higher inbreeding coefficient than wild adults, the nursery seedlings from Foret Nord show a >50% higher value still. Benign greenhouse environments have been associated with survival of individuals with high inbreeding coefficients in nursery stock of other tree species (Konnert and Ruetz, 2003).

In summary, both nursery populations show evidence of being genetically compromised, but in different ways. The nursery stock derived from Kaanua is genetically depauperate; the stock derived from Foret Nord does not suffer from low genetic diversity, but is inbred.

4.3. *Implications for conservation and forest restoration*

Populations of *A. nemorosa* are subject to considerable threats as a consequence of habitat degradation (Kettle et al., 2007). The conservation of this species to some extent is dependent on establishment of nursery reared seedlings. It is therefore vital that this stock is genetically robust.

Although the limited number of nursery populations available precluded a detailed replicated experiment on the efficacy of different collecting strategies, it is possible to make some common-sense observations based on the results obtained here. The most striking genetic compromise was found in nursery stock derived from cone collections from adult trees in Kaanua. Climbing individual trees is time consuming and requires specialist equipment limiting the potential to sample many trees. Furthermore, the high variance in cone production in *A. nemorosa* is likely to lead to sampling trees with large numbers of cones if maximising the number of seeds is the primary aim of seed collectors. Coupled with high variance in the seed viability of those cones that were sampled, these factors appear to have combined to result in a severe cryptic bottleneck. If direct cone collection is used for establishing future nursery collections, tracking the progeny sampled from different mother trees would be a practical method of identifying situations where nursery stock is becoming dominated by a small number of families. Sampling over multiple years would also help to maximise the number of trees contributing to the nursery stock.

In contrast to the direct collection of cones, the nursery stock from Foret Nord was collected directly from the forest floor. There was no evidence of a genetic bottleneck in this sample. Adopting this sampling approach is likely to result in a more comprehensive sample of seed from the entire population as it is easy to cover a wide area that contains seed

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dispersed from many trees. However, as this seed showed a higher level of inbreeding than that in the wild seedling population, culling of inferior stock in the nursery may be required.

One potential alternative future strategy for the establishment of nursery stock for this endangered conifer is sampling established wild seedlings directly from the forest floor and rearing these on in nurseries. The wild seedlings in both source populations contain levels of variation comparable to the adult populations and are likely to be the product of a much larger effective population of adults than would contribute to a single season's seed. Although the wild seedling populations are more inbred than the wild adults (Kettle et al., 2007), these seedlings will already have been subject to some degree of selection (Table 3), and some highly inbred seeds will have been selected out. The fitness of established seedling populations is therefore predicted to be higher than that of the seed population. As mortality levels in *A. nemorosa* seedlings are high especially in old growth forests because of competition from angiosperm species (Kettle, 2006), collection of some of these seedlings may therefore represent an efficient use of this material. This of course needs balancing against the risk of negatively affecting any residual natural regeneration that may occur, and the cost implications and practical challenges of translocating living seedlings. These considerations will place an upper limit on the sample sizes that can be collected in this fashion.

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