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**Genetic structure of East Antarctic populations of the moss Ceratodon purpureus**

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Abstract
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Keywords
bryophytes, clonal diversity, dispersal, microsatellites, RAPDs, Windmill Islands.

Disciplines
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Genetic structure of East Antarctic populations of the moss *Ceratodon purpureus*

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**Abstract:** The capacity of the polar flora to adapt is of increasing concern given current and predicted environmental change in these regions. Previous genetic studies of Antarctic mosses have been of limited value due to a lack of variation in the markers or non-specificity of the methods used. We examined the power of five microsatellite loci developed for the cosmopolitan moss *Ceratodon purpureus* to detect genetically distinct clones and infer the distribution of clones within and among populations from the Windmill Islands, East Antarctica. Our microsatellite data suggest that the extraordinarily high levels of variation reported in RAPD studies were artificially elevated by the presence of contaminants. We found surprisingly little contribution of asexual reproduction to the genetic structure of the Windmill Islands populations, but more loci are required to determine the distribution of individual clones within and among populations. It is apparent that Antarctic populations of *C. purpureus* possess less genetic diversity than temperate populations, and thus have less capacity for adaptive change in response to environmental variation, but more markers are needed to resolve the total genetic diversity in Antarctic *C. purpureus* and other mosses.

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**Introduction**

The climates of the Arctic and West Antarctica, including the Antarctic Peninsula, have warmed over the past 50 years to temperatures unprecedented in recent geological time (Briffa *et al.* 1995, Luckman 1998, Domack *et al.* 2005). In addition, Antarctica has experienced the largest increase in UV radiation as a result of stratospheric ozone depletion beginning in the 1970s (Madronich *et al.* 1998). In the Windmill Islands region, long-term drying as a result of isostatic uplift may be accelerated by more recent climate change (Goodwin 1993, Hodgson *et al.* 2006). This has raised concerns regarding the ability of the polar biota to cope with this rapid rate of environmental change. As the genetic diversity of a population is expected to be a strong predictor of its capacity for adaptive change in response to environmental variation (Frankham 2005), quantifying the level of genetic variation in populations of Antarctic organisms is of critical importance.

The polar flora is dominated by cryptogams (mosses, liverworts, lichens and algae), particularly in Antarctica where vascular plants are currently restricted to the comparatively mild Antarctic Peninsula. The frequency of sexual reproduction in mosses tends to decrease with increasing latitude, such that sexual reproduction becomes increasingly rare in the sub-Antarctic and Arctic, and most Antarctic mosses are considered to rely exclusively on asexual reproduction (Longton 1988, Smith & Convey 2002). As most Antarctic mosses exist solely as haploid gametophytes and are restricted to small, isolated areas of ice free habitat, their life history is predicted to result in relatively low levels of genetic variation (Stevens *et al.* 2007, Clarke *et al.* 2008), potentially making Antarctic moss populations particularly susceptible to environmental change.

Previous attempts to estimate the genetic diversity of Antarctic moss populations were of limited value because of a lack of variation in the markers and the impact of sample contamination in studies using non-specific PCR-based approaches. Allozymes have provided most of the useful data but have been limited by either small numbers of loci or specimens, making it difficult to assess the true extent of genetic variation. A survey of 15 enzyme systems in three mosses from the Windmill Islands, East Antarctica yielded ten systems that produced scorable phenotypes, however no intraspecific variation was detected in the mosses *Ceratodon purpureus* or *Schistidium antarctici* (formerly *Grimmia antarctici*, Melick *et al.* 1994). In contrast, *Bryum pseudotriquetrum* from the same region showed variation for two allozyme loci, revealing at least five distinct genotypes from ten samples collected across the region (Melick *et al.* 1994). The authors inferred that *C. purpureus* and *S. antarctici* populations may have arisen from a single colonization event with subsequent dispersal, whereas *B. pseudotriquetrum* may have colonized the region multiple times. However, only 3–16 samples were analysed per species (Melick *et al.* 1994), thus the low variation observed may simply reflect the effect of limited sample size. A separate allozyme study of the moss *Sarconeurum glaciale* had a much larger sample size (n = 60.
across two regions, Vestfold Hills and southern Victoria Land, separated by 2700 km) but tested only five enzyme systems, and could only identify consistent variation between regions for one allozyme locus (Selkirk et al. 1997). Another three allozyme loci yielded zymograms suggestive of variation within regions, but could not be scored reliably due to weak banding and a lack of repeatability, thus the authors employed Random Amplified Polymorphic DNA (RAPD) markers to examine genetic variation in the two regions.

Markers that detect variation at the level of the DNA sequence rather than the amino acid sequence have become the preferred method to investigate genetic diversity in the last two decades due to the increased power to detect genetically distinct individuals. PCR-based methods may also reduce the material required per sample, minimizing the impact of sample collection on ecosystems. The RAPD technique has been applied to Antarctic populations of Ceratodon purpureus, Schistochila glaciale, B. pseudotriquetrum, Anomobryum subrotundifolium (referred to as B. argenteum), Hennediella heimi, Pohlia nutans and Campylopus pyriformis (reviewed in Skotnicki et al. 2000, 2002). In contrast to the low levels of genetic variation reported for allozymes, upwards of 20 distinct (but related) genotypes were detected within a single moss clump in studies using RAPD markers, and in some cases genetic variation was detected along the length of single shoots (Skotnicki et al. 2004). The high level of RAPD variation detected was claimed to reflect somatic mutation, possibly due to increased UV from ozone depletion. However, a study by Stevens et al. (2007) has shown that DNA from non-target fungal and protozoan organisms, detected in more than 80% of Antarctic moss samples tested, prevents the accurate measurement of genetic variation in mosses using RAPD markers. High levels of diversity detected using RAPDs may thus reflect the lack of species-specificity of these markers rather than the true genetic variation in Antarctic moss populations.

Microsatellite genotyping is the current technique of choice for most studies that require species-specific markers to estimate mating systems or partition variation within or among populations (Selkoe & Toonen 2006). Microsatellites are tandem repeats of 1–6 nucleotides and mutate frequently compared to other genomic regions, thus typically yielding high levels of variation. However, the application of this technique typically requires that microsatellite primers first be developed for the species of interest, limiting their immediate applicability for most moss species. Primers can be developed by screening a genomic clone library or alternatively by searching published DNA sequences. Ceratodon purpureus (Hedw.) Brid. has been used as a model organism for the study of plant growth responses, thus has more DNA sequence data available on GenBank than any other moss species present in Antarctica.

Ceratodon purpureus is a cosmopolitan moss species found on all continents as well as many islands (Burley & Pritchard 1990). In temperate regions, this species reproduces both sexually through the production of abundant wind-dispersed spores and asexually through the regeneration of vegetative fragments and branching. Ceratodon purpureus has never been observed to reproduce sexually in continental Antarctica (Selkirk 1984, Burley & Pritchard 1990, Skotnicki et al. 1998, Smith & Convey 2002), therefore populations in the Windmill Islands are assumed to be clonal. Reliance on asexual reproduction combined with the isolation of continental Antarctic C. purpureus populations appears to reduce the genetic diversity present compared to temperate populations, potentially limiting the capacity of Antarctic populations to adapt to environmental change (Frankham 2005, Clarke et al. 2008). We examined the power of five microsatellite loci developed from the available sequence data for C. purpureus (Clarke et al. 2008) to detect genetically distinct clones and infer their distribution within and among the Windmill Islands populations. We also compared the amount of genetic variation in populations from the Windmill Islands and the degree of clonality in
Antarctic *C. purpureus* with that in sexually reproducing populations from Wollongong, Australia.

### Methods

**Population sampling and molecular analysis**

*Ceratodon purpureus* was collected from six populations from the Windmill Islands region, East Antarctica (Fig. 1). We sampled clumps separated by a minimum of 1–2 m to reduce the chance of repeatedly sampling the same clone (referred to as independent samples). Sampling from the corners and centre of a 20 cm quadrat (maximum of five samples) was also performed 1–4 times in populations with sufficiently extensive colonies to examine the distribution of genetic variation at smaller scales. Quadrats contained either continuous or patchy *C. purpureus* colonies, interspersed with other mosses, moribund moss colonies or rocks. Based on a shoot density for Antarctic *C. purpureus* turfs of 900 cm\(^{-2}\) (Wasley *et al.* 2006), a quadrat could contain up to 360 000 individuals. Samples for a single population were collected within 500 m of each other. A similar combination of independent and quadrat sampling was used to collect *C. purpureus* from three populations in the Wollongong–Sydney region, Australia. DNA extraction from single shoots and microsatellite analysis, including PCR amplification and visualization of PCR products, was performed as described in Clarke *et al.* (2008).

**Statistical analysis**

Standard genetic parameters for each population were estimated using GenAlEx version 6.0 (Peakall & Smouse 2006). Observed genotypic diversity (*G*\(_O\)), and significant deviations from the expected genotypic diversity under conditions of sexual reproduction and random mating (*G*\(_E\)) were estimated for populations as described in Clarke *et al.* (2008). The number of clones detected per quadrat was compared between the Windmill Islands and Wollongong–Sydney regions using a *t*-test performed with JMP version 5.1. Data were inverse-transformed to satisfy the assumptions of normality and homoscedascity. The probability of multiple samples within a population sharing a common genotype arising from distinct sexual reproductive events (*P*\(_{sex}\)) was estimated using the expression

\[
P_{sex} = n^{-1}C_{r-1}(P_{gen})^{r-1}(1 - P_{gen})^{n-r}
\]

(Willis & Ayre 1985) where *n* is the number of samples in a population, *r* is the number of samples sharing a given genotype, and *P*\(_{gen}\) is the
probability of occurrence for that genotype (the product of the component allele frequencies). Ceratodon purpureus is dioecious and gametes have limited mobility. Sperm must swim from the male gametophyte to the female, and so are expected to travel only centimetres. Female archegonia remain fixed to the female gametophyte and so are immobile. We therefore treated each site as a potential breeding population and used allele frequencies for each site to estimate \( P_{\text{sex}} \). Furthermore, ramets from a single genet will be of the same sex, so mating can only occur amongst distinct genets, reducing the degree to which a large clone will contribute disproportionately to each sexual generation. Allele frequencies used to estimate \( P_{\text{sex}} \) were therefore calculated using two methods; firstly using the allele frequencies from only the novel genotypes in each population (assuming all genotypes contribute equally to the next generation), and secondly using the allele frequencies from the independent samples and novel genotypes in quadrat samples in each population (assuming more widespread genotypes contribute more to the next generation). The probability of samples sharing a common genotype arising in separate populations from distinct reproductive events was calculated as the product of \( P_{\text{gen}} \) for the genotype in each population it was detected in, using both methods for calculating \( P_{\text{gen}} \). Where multiple tests were performed \( P \)-values were adjusted using a sequential Bonferroni correction (Rice 1989).

The likelihood of each sample from the Windmill Islands representing a first-generation immigrant was calculated using GENECLASS2 (Piry et al. 2004). First-generation migrant detection was computed using the ‘likelihood of the individual genotype within the population where the individual has been sampled’ criterion (L_home). Monte Carlo resampling was used to compute a random sample of multilocus genotypes for 1000 individuals to generate assignment criterion values using a method designed to reduce the likelihood of assigning a resident genotype as a migrant (type I error, Paetkau et al. 2004). Four likelihood estimation criteria were employed; one distance-based approach (Nei’s standard genetic distance, Nei 1972), a frequency-based approach (Paetkau et al. 2004), and two Bayesian approaches (Ramula & Mountain 1997, Baudouin & Lebrun 2000). Biased allele frequencies due to widespread clones may increase the type I error rate, however low sample sizes would also increase the likelihood of type I error. Therefore three datasets were tested, all (108) samples, independent samples and novel quadrat genotypes (55 samples), and novel genotypes across the region (13 samples).

### Results

**Genetic variation and identification of clones**

Only four of the five microsatellite loci that were variable in temperate *C. purpureus* populations were polymorphic in samples from the Windmill Islands, and Windmill Islands populations generally supported less genetic diversity. The CEPU108 locus displayed 1–2 alleles per population and 3–4 alleles per region in *C. purpureus* from Australia and Finland, but was monomorphic in the Windmill Islands region. Variable loci displayed 2–4 alleles per locus in the Windmill Islands (Table I). There were no significant differences between the Windmill Islands populations in terms of numbers of alleles or expected heterozygosity per locus, regardless of whether quadrat samples were included or not (Fig. 2). The Red Shed population tended to be the least diverse in terms of number of alleles per locus and expected heterozygosity.
for both analyses. The Science Building and ‘Clark Ridge’ (unofficial name) had the highest expected heterozygosity and number of alleles per locus, respectively.

Reduced allelic diversity in the Windmill Islands compared to temperate populations (Clarke et al. 2008) means more loci are required to achieve the same power to detect genetically distinct individuals. The optimum number of loci to detect all possible genetically distinct clones can be determined by increasing the number of loci until additional (variable) loci no longer reveal any additional clones (Arnaud-Haond et al. 2005). A plot of the number of clones detected in the Windmill Islands with increasing numbers of microsatellite loci shows no evidence of approaching an asymptote (Fig. 3), indicating that these markers are most probably not resolving all clones in the region.

Clonal diversity within and among sites

Combining the microsatellite loci revealed a total of 13 distinct multilocus genotypes from the 108 Windmill Islands samples. The most common multilocus genotype in the Windmill Islands (genotype E, Table I) was found in 75 samples (69.4%) and was present in all populations (Table II). The Red Shed population had the lowest number of distinct genotypes (two), whereas the ‘Clark Ridge’, Science Building and Robinson Ridge populations had the most (five each). Seven samples possessed unique multilocus genotypes, two each at Robinson Ridge, Science Building and ‘Clark Ridge’, and one at Stevenson Cove (Table II). Of the 12 sets of quadrat samples (maximum of five samples each) from the Windmill Islands region, six were composed of a single multilocus genotype and four were 80% one genotype, with the most common genotype over the region also the most common in each of these quadrats. All quadrats contained only one or two genotypes, except a single quadrat from ‘Clark Ridge’ that contained four distinct genotypes.

The observed genotypic diversity (\(G_O\)) for the independent samples at each population ranged from 0.90 (Red Shed) to 3.38 (Science Building, Table III). Including quadrat samples reduced the observed genotypic diversity in all Windmill Islands populations, except ‘Clark Ridge’, which was not tested without quadrats due to only three independent samples, and the Red Shed population. Only Robinson Ridge (including quadrat samples) shows a significant deviation from genotype frequencies expected under conditions of sexual reproduction and random mating, but this value is no longer significant after applying a Bonferroni correction. In contrast, even after Bonferroni correction, we estimated that in five of the 11 instances where a single genotype occurred in multiple samples within a population, it was significantly \(P < 0.05\) unlikely that the observed level of replication reflected independent products of sexual reproduction. More conservative estimates using allele frequencies from all independent samples in each population gave four genotypes unlikely to be present at the observed frequency due to independent sexual reproductive events, but only one following a Bonferroni correction.

The distribution of multilocus genotypes suggests that dispersal between sites does occur in the Windmill Islands region. In addition to the most common multilocus genotype being present at all sites, five other genotypes (A–D and F) were shared among 3–10 samples each and were present in 2–3 sites, with the exception of genotype C which was only found in the Science Building population. Indeed, more than half the samples from the Science Building population (eight of 14) represented genotypes not found anywhere else in the Windmill Islands region. Widespread genotypes detected in multiple populations may reflect dispersal between sites or simply a lack of power to resolve all clones. However, the probability of genotypes common to multiple populations arising in each population by distinct reproductive events was < 0.05 for four of five genotypes in which this occurred using allele frequencies from independent samples in each population, and < 0.01 for two of these, with similar results using the allele frequencies from the novel genotypes. It is thus probable that the presence of identical genotypes in multiple populations represents some dispersal of clonal fragments between sites.

Assignment test for first generation immigrants

One of the unique genotypes from ‘Clark Ridge’ (genotype M) possessed two alleles not present in any other Windmill Islands population, suggesting it may have originated from a different source. Indeed, this sample was identified as a first-generation migrant in 11 of the 12 combinations of datasets and likelihood estimation criteria, the exception being the distance-based approach using the novel genotypes dataset. Simulations of the assignment methods used here show that approximately 50 diploid individuals are required to minimize the rate of type I error (resident individuals incorrectly assigned as immigrants, Paetkau et al. 2004). Assuming a diploid individual provides twice the information as a haploid individual, only the dataset including all sampled individuals may be sufficiently large in this study. Only one other genotype (Robinson Ridge, genotype J) was identified as a potential migrant with any method (distance-based approach with all samples).

Comparison of clonal diversity in Antarctic and temperate sites

Ceratodon purpureus populations from the Wollongong–Sydney region showed much higher levels of clonal diversity than the Windmill Islands populations. In comparison to the 13 distinct multilocus genotypes from
the 108 Windmill Islands samples, 51 multilocus genotypes were identified from 94 samples in the Wollongong–Sydney region. Whereas the 12 sets of quadrat samples from the Windmill Islands region were predominantly composed of a single multilocus genotype, all but one of the 12 quadrats from the temperate Wollongong populations contained more than one genotype, and there were significantly more distinct genotypes per quadrat in the Wollongong–Sydney populations (2.8 ± 0.3) compared to the Windmill Islands (1.7 ± 0.3 genotypes per quadrat, \( t = 2.87, P = 0.009 \)).

**Discussion**

Microsatellites proved useful in analysing the genetic structure of both Antarctic and temperate populations of *C. purpureus* and somewhat surprisingly revealed lower than predicted levels of clonal replication and much lower levels of genetic diversity than reported using RAPDs (Skotnicki et al. 2004). Overall, our data imply that Antarctic populations contain relatively little genetic variation, making them potentially vulnerable to climate change.

There is some evidence from allozyme studies that Arctic moss populations possess less genetic diversity than temperate moss populations. Fifteen loci were scored from ten enzyme systems in an allozyme study of *Sphagnum* species from Arctic Svalbard, but no variation was detected in four *S. tundræ* populations (\( n = 10–16 \) samples per population), and only four haplotypes were detected in *S. teres* over three populations (\( n = 8–10 \)), with no intrapopulation variation in two of these (Flatberg & Thingsgaard 2003). A similar allozyme study of more temperate *S. teres* populations by Cronberg (1996) revealed more than twice the number of haplotypes per population (3.2) and proportion of distinguishable genotypes (PD, number of clones detected divided by the sample size - 0.47) than in Arctic populations (1.33 haplotypes per population, PD - 0.15). Similarly, a survey of 18 allozyme loci in boreal, sub-Arctic and Arctic populations of the moss *Meesia triqueta* found a significant decrease in genetic diversity with increasing latitude (Montagnes et al. 1993).

Allelic diversity did not vary significantly between populations in the Windmill Islands, however some trends were apparent. The tendency for the Red Shed population to show lower genetic variation than other Windmill Islands populations may reflect disturbance during the construction of Casey Station in the early 1980s, as this site is directly adjacent to the accommodation building. In contrast the Science Building population shows the highest expected heterozygosity. This site showed evidence of contamination from cement dust from ongoing construction work at Casey when samples were collected (Adamson et al. 1994). More than half the samples from the Science Building population were genotypes not found anywhere else in the Windmill Islands region, thus it is possible that these genotypes may be more tolerant of this stressor, or may represent recent introductions (Frenot et al. 2005). Genetic variation also tended to be higher in drier sites (Robinson Ridge, ‘Clark Ridge’, Science Building) than wetter sites (Red Shed and ASPA 135, Lovelock & Robinson 2002, Wasley et al. 2006). It is possible that meltwater at wet sites facilitates the dispersal of asexual fragments and the spread of clones. More markers and comparable sampling between sites is required to determine whether genetic diversity is influenced by site characteristics such as water availability or level of disturbance.

Despite the apparent absence of sexual reproduction in continental Antarctic *C. purpureus* populations, comparing the observed genotypic diversity with that expected of sexual populations showed no deviation from random mating, even when quadrat samples (likely to contain more replicates of asexually produced individuals) were included, with the possible exception of Robinson Ridge. Our data thus suggest that these populations have either retained diversity from past episodes of sexual reproduction (Hsiao & Rieseberg 1994, Sherman et al. 2006), or are continuing to receive input of sexually produced spores. Several palaeoclimate records suggest warmer conditions than at present in East Antarctica around 3000 yr BP (reviewed in Hodgson et al. 2004), which could have led to a greater incidence of sexual reproduction. However, genetic evidence suggests intercontinental migration of *C. purpureus* is relatively common, presumably via spores thought to be capable of dispersing thousands of kilometres (van Zanten 1978, McDaniel & Shaw 2005), thus it is also possible that spores could arrive in continental Antarctica from temperate or sub-Antarctic populations (Smith 1991). A single sample from the ‘Clark Ridge’ population possessed two alleles not found in any other sample from the Windmill Islands region, and was consistently identified as a recent migrant to the population using probability of residence calculations. Although the frequency of pollen deposition in moss turfs from the Windmill Islands was 100-fold lower than in turfs from South Shetland Islands near the tip of the Antarctic Peninsula (Kappen & Straka 1988), the presence of pollen indicates some degree of connectivity between the Windmill Islands and more temperate regions. In contrast to the claim that *C. purpureus* populations in the region may have arisen from a single colonization event with subsequent dispersal (Melick et al. 1994), the presence of this putative migrant suggests that, despite the isolation of the Windmill Islands, *C. purpureus* may be continuing to arrive and become established in these populations. Although assignment tests as used here are often useful for identifying immigrants to an otherwise static population with short sexual generations, in this case populations may be maintained for extremely long periods by somatic growth and fragmentation. As our results indicate that the diversity of genotypes detected
within populations reflects past sexual reproductive events either within the Antarctic or a distant source population, the outlier genotype may simply have a different origin to the majority of the population.

There are indications from this study that asexual reproduction is contributing to the genetic structure of *C. purpureus* populations from the Windmill Islands. At least one population contains multiple samples that share a common genotype with a low probability of occurring at the observed frequency by distinct sexual reproductive events. The predominance of a single genotype within each 20 cm quadrat in the Windmill Islands region suggests that somatic growth and fragmentation may be more important for colony expansion at this scale in the Antarctic than in temperate Australian populations, where colonies separated on the scale of centimetres often represent genetically distinct clones. Furthermore, expected genotypic diversity estimates used to detect deviations from random mating are sensitive to the number of samples and loci used and skewed allele frequencies (Stoddart & Taylor 1988). Most microsatellite loci in this study have skewed allele frequencies (< 0.2 or > 0.8) in each population, and the proportion is increased when quadrat samples are included. Skewed allele frequencies can cause predicted diversity to be lowered to the point that asexual reproduction has little impact, such that clonal and panmictic populations show similar levels of genotypic diversity (Stoddart & Taylor 1988). More markers are required to determine the extent of clones and the contribution of asexual reproduction to genetic structure of *C. purpureus* from the Windmill Islands.

Although genotypes shared amongst separate populations are less likely to represent the same clone as genotypes shared within a population, the low probability of the multilocus genotypes present at multiple sites arising by separate reproductive events suggests that dispersal of asexual fragments between populations is occurring in the Windmill Islands. Populations from Clark Peninsula and Robinson Ridge are separated by c. 13 km, thus genotypes shared amongst these locations suggest that propagules are able to disperse and colonize sites separated at such a scale. Whether asexual propagules are capable of colonizing more widely separated regions of continental Antarctica, or whether this requires the input of sexually produced spores better suited to long distance dispersal from the sub-Antarctic or temperate regions, remains unknown.

**Conclusion**

Microsatellites reveal greater genetic diversity in Antarctic *C. purpureus* populations than has been detected with allozymes, but suggest that the extraordinarily high levels of variation reported for RAPD studies were artificially elevated by the presence of contaminants. Asexual reproduction appears to make surprisingly little contribution to population structure, but more loci are required to determine the extent to which individual clones are distributed within and among populations. Antarctic populations of *C. purpureus* possess less genetic diversity than temperate populations, and thus have less capacity for adaptive change in response to environmental variation, but more markers are needed to resolve the total genetic diversity within and among regions in Antarctic *C. purpureus* and other mosses.

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