Annals of Botany **108**: 1247–1256, 2011
doi:10.1093/aob/mcr220, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

Low but structured chloroplast diversity in *Atherosperma moschatum* (Atherospermataceae) suggests bottlenecks in response to the Pleistocene glacials

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Received: 19 March 2011 Returned for revision: 2 June 2011 Accepted: 4 July 2011 Published electronically: 18 August 2011

• *Background and Aims* The cool temperate rainforests of Australia were much reduced in range during the cold and dry glacial periods, although genetic evidence indicates that two key rainforest species, *Nothofagus cunninghamii* and *Tasmannia lanceolata*, survived within multiple locations and underwent only local range expansions at the end of the Last Glacial. To better understand the glacial response of a co-occurring but wind-dispersed and less cold-tolerant rainforest tree species, *Atherosperma moschatum*, a chloroplast phylogeographic study was undertaken.

• *Methods* A total of 3294 bp of chloroplast DNA sequence was obtained for 155 samples collected from across the species' range.

• *Key Results* The distribution of six haplotypes observed in *A. moschatum* was geographically structured with an inferred ancestral haplotype restricted to Tasmania, while three non-overlapping and endemic haplotypes were found on the mainland of south-eastern Australia. Last glacial refugia for *A. moschatum* are likely to have occurred in at least one location in western Tasmania and in Victoria and within at least two locations in the Great Dividing Range of New South Wales. Nucleotide diversity of *A. moschatum* was lower ($\pi = 0{\cdot}00021$) than either *N. cunninghamii* (0·00101) or *T. lanceolata* (0·00073), and was amongst the lowest recorded for any tree species.

• *Conclusions* This study provides evidence for past bottlenecks having impacted the chloroplast diversity of *A. moschatum* as a result of the species narrower climatic niche during glacials. This hypothesis is supported by the star-like haplotype network and similar estimated rates of chloroplast DNA substitution for *A. moschatum* and the two more cold tolerant and co-occurring species that have higher chloroplast diversity, *N. cunninghamii* and *T. lanceolata*.

Key words: *Atherosperma moschatum*, *A. moschatum* subsp. *integrifolium*, Atherospermataceae, bottleneck, comparative phylogeography, glacial refugia, *Nothofagus cunninghamii*, Pleistocene glacials, south-eastern Australia, *Tasmannia lanceolata*, cool temperate rainforest.

INTRODUCTION

Understanding the ability of species to occupy newly available habitat is fundamental to predicting how species respond to environmental change. The dramatic environmental changes that have occurred since the Last Glacial Maximum (LGM) approx. 18 000 years ago provide a model for developing such an understanding. Worldwide, most areas of temperate forest vegetation are believed to have been established since the onset of favourable climates approx. 13 000 years ago through expansion from refugia in which they survived the cold and, in many regions, dry LGM conditions. The rate and the degree of colonization of the newly available habitat is largely considered to have been determined by individual responses of species (Davis, 1976; Huntley and Birks, 1983; Huntley and Webb, 1989) related to the number and location of glacial refugia, the individual fitness of species, and to life-history traits, including the mechanism of seed dispersal.

Australia's cool temperate rainforests provide an interesting system in which to investigate these processes. These evergreen, closed-canopy forests are widespread but discontinuously distributed within the continent's mountainous south-east corner and the island of Tasmania (Hill *et al.*, 1988). These forests are species poor, especially in trees, but contain many lineages with fossil histories extending back to the mid-Tertiary (30 mya) or earlier (Carpenter and Jordan, 1997; Hill, 2004; Sniderman and Jordan, 2011). Furthermore, fossil evidence indicates that almost all contemporary species have been present at least since the Early Pleistocene (2·5 million years to 788 000 years ago), and therefore have remained relatively unchanged through many, or perhaps all, of the glacial-interglacial cycles of the last few million years (Jordan, 1997). However, only recently has clear evidence for the location of glacial refugia and the geographic extent of post-glacial recovery for individual species of the cool temperate rainforest biome become available. Chloroplast phylogeographic evidence has shown that at least some of the species were highly resilient in response to the major climate changes of the Pleistocene. Chloroplast phylogeographies provide clear evidence for geographic stasis and glacial survival within multiple refugia for three species in Australian cool temperate rainforests: the gravity-dispersed

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dominant tree species, *Nothofagus cunninghamii* (Worth *et al.*, 2009) and *Eucalyptus regnans* (Nevill *et al.*, 2010); and the fleshy-fruited shrub, *Tasmannia lanceolata* (Worth *et al.*, 2010). Evidence from a fourth species, the gravity- or water-dispersed conifer *Lagarostrobos franklinii* (Clark and Carbone, 2008), is sparser but is also consistent with stasis.

This study investigates the chloroplast phylogeography of *Atherosperma moschatum* (Atherospermataceae), a tree species for which there are several reasons to suspect a divergent response to the Pleistocene climatic perturbations compared with other Australian cool temperate rainforest species. *Atherosperma moschatum* has the most extensive latitudinal range of any Australian cool temperate rainforest woody species and has plumose, wind-dispersed seeds, that have been shown to have efficient dispersal over 150 m distance (Hickey *et al.*, 1982). The extensive geographical distribution of *A. moschatum*, particularly in contrast to the gravity-dispersed *N. cunninghamii* (a species that co-occurs extensively with *A. moschatum* in Tasmania and the highlands of Victoria), has been considered to have been facilitated by the greater ability of the species to disperse during the Holocene over areas of unsuitable habitat (Hickey *et al.*, 1982; Read and Busby, 1990; Neyland and Brown, 1993). For example, the occurrence of *A. moschatum* in eastern Gippsland of Victoria and in some small, topographically protected sites in the dry eastern half of Tasmania, where *N. cunninghamii* is absent, has been considered to be a consequence of the ability of *A. moschatum* to reach suitably wet isolated habitat via long-distance dispersal (Gilbert, 1959; Howard and Ashton, 1973; Neyland and Brown, 1993). Furthermore, physiological evidence suggests that *A. moschatum* is the least cold tolerant of all Australian cool temperate rainforest trees (Read and Hill, 1989; Read and Busby, 1990; Feild and Brodribb, 2001) and, as such, is a less likely candidate to have withstood glacial conditions in multiple regions. In addition, although drought tolerance has been investigated in only a small number of Australian cool temperate rainforest trees, *A. moschatum* was the most vulnerable to leaf hydraulic conductance failure during drought compared with three other widespread trees, *N. cunninghamii*, *Pittosporum bicolor* and *T. lanceolata* (Blackman *et al.*, 2010). Currently, there is little evidence available to discern the roles of either multiple glacial refugia or dispersal in explaining the widespread distribution of *A. moschatum*, largely because the fossil record is uninformative on this matter. Fossil pollen of *A. moschatum* is rare, with the only LGM records being from some parts of western Tasmania (Macphail and Colhoun, 1985; Colhoun *et al.*, 1999; Hopf *et al.*, 2000), although trace quantities have been found in the Australian Alps at approx. 32 000 years before present (Kershaw *et al.*, 2007; see Supplementary Data Fig. S1, available online). A previous genetic study of 22 populations based on isozymes suggested that populations in south-eastern Tasmania were closely related, while the three Victorian populations had different affinities, either nested within Tasmanian variation or closest to the most northern populations in New South Wales (NSW) (Shapcott, 1994). These most northern populations of *A. moschatum* in the Blue Mountains and Barrington Tops regions (Figs 1 and 2) were the most distinctive in the species. This finding suggests long term isolation of these northernmost populations (Floyd, 1990; Shapcott, 1994). Populations of the species in these two areas have been classified as a separate subspecies, subsp. *integrifolium*, mainly based on leaf morphology (Schodde, 1969; Foreman and Whiffen, 2007). A later study of leaf oil chemistry in the genus broadened the range of *A. moschatum* subsp. *integrifolium* to include a previously unstudied population at Monga approx. 200 km to the south of the Blue Mountains, NSW (Brophy *et al.*, 2009).

This study aimed to identify the responses of *A. moschatum* to past climatic perturbations by investigating the species' range-wide chloroplast DNA (cpDNA) phylogeography. Specifically, this study assessed whether the species responded to the Pleistocene glacials via local expansion from multiple glacial refugia or, alternatively, with widespread colonization during the Holocene via wind dispersal.

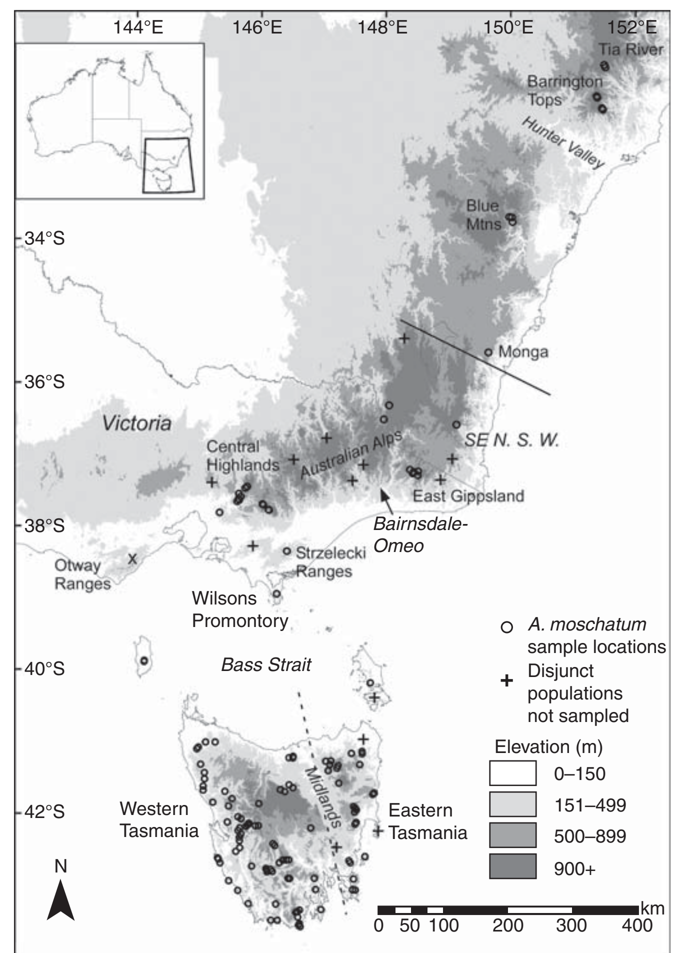

FIG. 1. Sample locations of *Atherosperma moschatum* (black circles, in some cases representing two or three plants; Fig. 2 shows the species' distribution). Some major geographic features, including areas of dry unsuitable habitat, are labelled in italics. The continuous line indicates the southernmost known extent of the subspecies, *Atherosperma moschatum* subsp. *integrifolium*. The broken line indicates the demarcation of eastern and western Tasmania through the dry Midlands of the island. The × indicates where the species occurred in the Otway Ranges until at least the 1860s and where it is now extinct (Lunt, 1992).

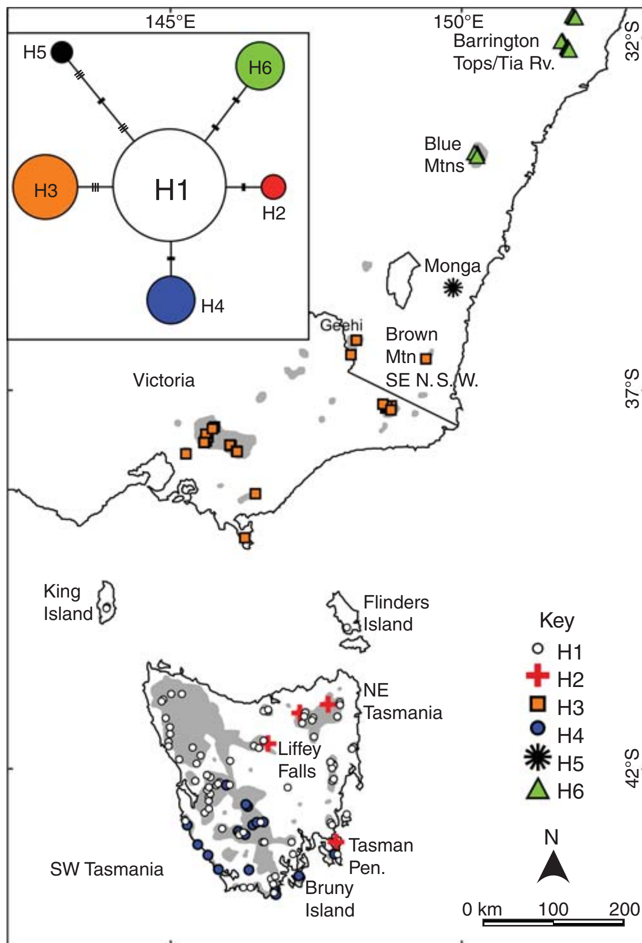

FIG. 2. The inset shows the median-joining network of the six haplotypes observed across the range of *Atherosperma moschatum*. The area of the circles in the haplotype network is proportional to the frequency of each haplotype. Lengths of lines connecting each haplotype are proportional to the number of character differences between them. Single nucleotide polymorphisms (SNPs) are indicated by solid bars and simple sequence repeats (SSRs) are indicated by three stacked lines. The main figure shows the distribution of the six haplotypes observed in *A. moschatum* (H1–H6). Grey areas indicate the natural distribution of the species [derived from the Natural Values Atlas (Department of Primary Industries and Water, Tasmanian Government), Australia's Virtual Herbarium (www.ersa.edu.au/avh/), Floyd (1989, 1990), Coyne (2001) and Ashton (2000)].

MATERIALS AND METHODS

The study species

Atherosperma moschatum Labill. (southern or black sassafras) is endemic to mainland south-eastern Australia and the island of Tasmania, and is the only species of its genus. This dioecious evergreen tree species can reach 45 m in height (Curtis, 1993). In Tasmania, *A. moschatum* is common from sea-level to approx. 900 m a.s.l., but does occasionally occur as a shrub at higher altitudes (Read and Hill, 1988*a*). On mainland Australia, the species is confined to mountainous areas, above 340 m a.s.l. in Victoria, 700–1000 m a.s.l. in south-eastern NSW, and generally above 1200 m a.s.l. in the northern part of its range at Barrington Tops and Tia River (Floyd, 1990), where the species has a very localized occurrence (Read and Hill, 1989). Across its 12° range in latitude (Read and Hill, 1988*a*), *A. moschatum* occurs within cool temperate rainforest, often as a subdominant to *Nothofagus* species. However, it is the sole cool temperate rainforest tree in parts of eastern Tasmania (Kirkpatrick, 1981; Neyland and Brown, 1993), in the Dandenong Range of Victoria (Howard and Ashton, 1973) and in localized parts of the Australian Alps (Gellie, 2005). It can also extend into the sub-canopy of wet eucalyptus forest (Gilbert, 1959; Ashton, 2000). In one location, the Otway Ranges of western Victoria (Fig. 1) where *N. cunninghamii* is the sole dominant rainforest tree, *A. moschatum* was present in the region during the Holocene until it went extinct after European settlement, with the last record of the species in the early 1860s (Lunt, 1992; McKenzie and Kershaw, 1997).

Sampling

Leaves were sampled from 155 *A. moschatum* individuals representative of virtually the whole species' geographic range (Fig. 1). In regions where the distribution of *A. moschatum* was more or less continuous (e.g. western Tasmania and the central highlands of Victoria) individuals were collected a minimum of approx. 1 km apart to maximize geographic coverage. Within some small and disjunct populations two or three samples were taken where ever possible. In total, 20 individuals of the northern subspecies, *A. moschatum* subsp. *integrifolium* (A.Cunn. ex Tul.) Schodde (Schodde, 1969; Foreman and Whiffen, 2007; Brophy *et al.*, 2009), and 135 individuals of the southern subspecies, *A. moschatum* subsp. *moschatum*, were sampled (see Supplementary Data Table S1).

For use as outgroups, one species each of three other genera of Atherospermataceae were sampled. These were the Chilean endemic *Laurelia sempervirens* (Ruiz & Pav.) Tul. (grown in a private collection by Ken Gillanders, southern Tasmania), *Nemuaron vieillardii* (Baill.) Baill. (from Mont Dzumac, New Caledonia) and *Daphnandra tenuipes* Perkins (from the Border Ranges National Park, north-eastern NSW).

Molecular methods

Total genomic DNA was extracted from 0·25 g of adult leaves using the Qiagen DNeasy Plant Mini Kit (Qiagen Pty Ltd, Vic, Australia). DNA quantity and quality were assessed by agarose gel electrophoresis with ethidium bromide staining and comparison with a standard molecular weight marker (lambda *Hin*dIII).

Eight primer pairs were trialled in a preliminary search for cpDNA variation via direct sequencing within 24 samples representative of most of the species range (for fragment names, primer pairs and references see Supplementary Data Table S2). PCR conditions were as follows: for *atp*B-1-*rbc*L-1, an initial 4 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 1 min and 15 s at 55 °C and an extension for 1 min and 15 s at 72 °C, and a final extension step for 10 min at 72 °C; for K1-*mat*K1 and *mat*K6-K2, an initial 4 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 1 min at 51 °C and extension for 1·5 min at 72 °C, and a final extension step for 10 min at 72 °C; for *psb*M-*trn*D, *pet*N-*psb*M and a-b reactions, an initial

4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 2 min at 51 °C and extension for 2 min at 72 °C, and a final extension step for 10 min at 72 °C; for e-f and c-d, an initial 1 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 50 °C and extension for 45 s at 72 °C, and a final extension step for 7 min at 72 °C. Quality of PCR products was assessed by gel electrophoresis on 1·2 % agarose gels and staining with ethidium bromide. In preparation for DNA sequencing, PCR products were purified using the Qia-Quick PCR purification kit (Qiagen Pty Ltd). DNA sequencing was performed in one direction using the forward primers K1, *mat*K6, *psb*M2, a and c except for the *atp*B-*rbc*L and *pet*N-*psb*M fragments which were sequenced in both directions.

The *trn*L-*trn*F fragment did not amplify, a finding also observed by Renner *et al.* (2000) for *A. moschatum*. Since no variation was observed within the *psb*M-*trn*D and *atp*B-*rbc*L fragments, only the K1-*matK1*, *mat*K6-K2, *petN-psb*M, *trnT-trnL* and the *trn*L intron fragments were sequenced for all 155 samples.

Sequencing reactions were performed in an MJ Research PTC-225 thermal cycler using ABI prism Bigdye terminator v 3·0 cycle sequencing kits (Applied Biosystems, CA, USA) with AmpliTaq DNA polymerase (Applied Biosystems) and fragment separation was undertaken on a 3730*xl* DNA analyser (Applied Biosystems). Sequences were aligned using Sequencher 4·5 (Gene Codes Corporation, MI, USA), and checked by eye for incorrect base calls, single nucleotide polymorphisms (SNPs), DNA insertions and deletions (indels) and simple sequence repeat (SSR) variations.

Haplotype network and phylogenetic analyses

For all *A. moschatum* haplotypes, a median-joining network, with equal weighting of all characters, was constructed using Network 4·5·0·2 (Bandelt *et al.*, 1999). To polarize the haplotype relationships a phylogenetic analysis of all haplotypes, including the outgroups (*Laurelia sempervirens*, *Nemuaron vieillardii* and *Daphnandra tenuipes*), was undertaken using exhaustive searches for most-parsimonious trees with PAUP* version 4·0b10 (Swofford, 2000). The non-overlapping forward and reverse sequences for the *petN-psb*M intergenic spacer were concatenated into a single alignment for these and all subsequent analyses. Indels were scored as binary characters, while SSR regions were scored as multistate characters with a maximum of four states. All characters, including indels and SSR regions, were treated as unordered and of equal weight. Eight bases of the *trn*T-*trn*L intron were excluded from the analysis as the alignment of this region was ambiguous. Branch support was assessed by bootstrap analysis (Felsenstein, 1985) with 1000 bootstrap replicates using the same search parameters as those in the parsimony analysis. The K1-*mat*K1 and *pet*N-*psb*M sequences were of poor quality for the outgroup *D. tenuipes* and were scored as missing data.

DNA sequence based analyses and comparison with other species

Nucleotide diversity (π; Nei, 1987), the average number of nucleotide differences per site (excluding indels) between any two randomly chosen DNA sequences, of *A. moschatum* was calculated with the program DnaSP version 5·10 (Librado and Rozas, 2009) separately for each cpDNA fragment alignment (*pet*N-*psb*M, *trn*L-*trn*T, *trn*L intron, K1-*mat*K1 and matK6-K2) and for a concatenated alignment of all fragments. For comparison, the DNA sequence datasets of *Nothofagus cunninghamii* (213 samples; 2164 bp; *trn*S-*trn*f_M, *trn*L-*trn*F, *psbM-trn*D, *pet*N-*psb*M) from Worth *et al.* (2009) and *Tasmannia lanceolata* (244 samples; 3206 bp; *pet*N-*psb*M, *psb*M-*trn*D, *trn*L-*trn*F, *trn*L intron, K1-*mat*K1 and *mat*K6-K2) from Worth *et al.* (2010) were also analysed. Alignments were constructed with MEGA 4·0 implementing the ClustalW program (Tamura *et al.*, 2007). In addition, indel diversity per site [π(I)] was analysed for each species, with the multi-allelic option selected, using DnaSP version 5·10 (Librado and Rozas, 2009).

The distribution of pairwise mismatches (i.e. SNP differences between sequences, excluding indels) between cpDNA haplotypes was investigated using DnaSP version 5·10 (Librado and Rozas, 2009). For each species, this was undertaken using four different datasets; the whole species sampled range, and samples from three major regions, western Tasmania, eastern Tasmania and mainland Australia. Eastern Tasmania was defined as the region east of a line passing through the centre of the Midlands (Fig. 1) and extending into the Bass Strait, while western Tasmania was defined as being west of this line. This separation is based on the presence of the apparently significant biogeographic barrier formed by the broad, low, dry valley of the Midlands, and is consistent with phylogeographic structuring in *N. cunninghamii* and *T. lanceolata* (Worth *et al.*, 2009, 2010).

Estimates of cpDNA substitution rates

The nucleotide substitution rates per million years (SSMY) were estimated for concatenated alignments by calculating the mean number of nucleotide substitutions per site (Nei, 1987) with Jukes and Cantor (1969) correction between all ingroup sequences and outgroups using DnaSP version 5·10 (Librado and Rozas, 2009). The outgroups were *Laurelia sempervirens* and *Nemuaron viellardii* for *A. moschatum*, *Drimys winteri* and *Pseudowintera colorata* for *T. lanceolata*, and *N. glauca* and *N. menziesii* for *N. cunninghamii*, with sequence data for *Tasmannia* and *Nothofagus* from Worth *et al.* (2010) and Worth *et al.* (2009), respectively. Estimated dates of divergence (mya) of ingroups and outgroups taxa were obtained from fossil calibrated molecular based phylogenies from Renner (1999) and Renner *et al.* (2000) for Atherospermataceae, Marquínez *et al.* (2009) for Winteraceae and, for *Nothofagus* subgenus *Lophozonia*, Cook and Crisp (2005) and Knapp *et al.* (2005).

RESULTS

Chloroplast variation

Five SNPs (two transitions and three transversions) and three 1-bp indels (for description of *A. moschatum* chloroplast polymorphisms see Supplementary Data Table S3) were observed within the 3294 bp of aligned sequence obtained for all 155 *A. moschatum* samples. All indels were associated with A or T mononucleotide repeats (SSRs) over 10 bp in length.

Aligned sequence lengths and GenBank accession numbers for each fragment used in the preliminary screening and full sample set are shown in Supplementary Data Table S4.

Phylogenetic relationships

The eight polymorphisms observed in *A. moschatum* defined six haplotypes which formed a haplotype network without loops (Fig. 2). Parsimony analysis (using the outgroups and all *A. moschatum* haplotypes) resulted in four trees (36 characters parsimony informative; length = 142; consistency index = 0·99; retention index = 0·97) (Fig. 3). Bootstrap support (BP) was high for the monophyly of *A. moschatum* (BP = 100 %; Fig. 3) and for a clade containing *L. sempervirens* and *N. vieillardii* (BP = 97 %).

Geographic distribution of haplotypes

The six haplotypes observed in *A. moschatum* showed geographic structure and generally occupied separate regions across the species latitudinal range. In Tasmania, three haplotypes (H1, H2 and H4) were observed among the 106 samples. Haplotype H1 (55 % of all *A. moschatum* samples) was widespread including the King and Flinders Islands in Bass Strait, but was not found among the 49 samples from mainland Australia (Fig. 2). Haplotype H2 was rare (2·6 % of all samples) and was observed in north-east Tasmania ($n = 2$), north-central Tasmania (Liffey Falls; $n = 1$) and south-eastern Tasmania (Tasman Peninsula; $n = 1$). Haplotype H4 (10·3 % of all samples) was observed only in central and south-west Tasmania, Bruny Island and the Tasman Peninsula. This latter site contained all three Tasmanian haplotypes (Fig. 2), making it the most diverse region sampled. Haplotype H3 (18·7 % of all samples) was the only haplotype observed in Victoria and in south-eastern NSW at Brown Mountain and the Geehi area, Kosciusko National Park (Fig. 2). Samples of the northern subspecies, *A. moschatum* subsp. *integrifolium*, harboured two different haplotypes differing by five polymorphisms. All three samples from the isolated population at Monga National Park possessed haplotype H5 (1·9 % of all samples), which was not found anywhere else. The 17 samples from three isolated populations in the northern parts of the range shared haplotype H6 (11 % of all samples). Overall, four of the haplotypes (H1, H2, H3 and H6) transgressed some major areas of unsuitable habitat, the Midlands of Tasmania for H1 and H2, parts of Bass Strait for H1, the rain shadow of the Bairnsdale-Omeo corridor (Howard and Ashton, 1973) for H3 and the Hunter Valley in NSW for H6.

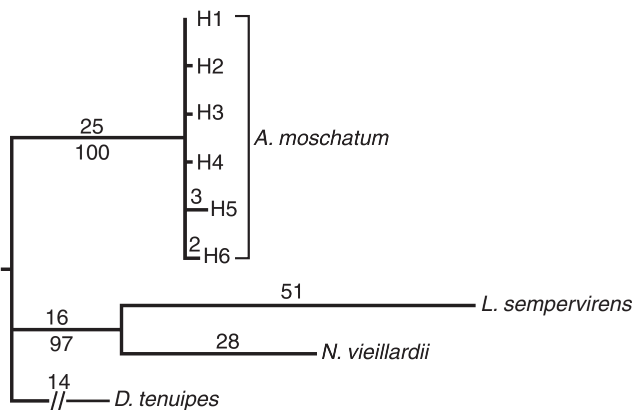

FIG. 3. One of four most-parsimonious trees obtained from chloroplast sequence characters in *Atherosperma moschatum* and outgroups. This tree is identical to the strict consensus. Branch lengths (the inferred number of single base pair substitutions and indels on a branch) greater than one are indicated above branches while bootstrap values above 50 % are shown below branches. Names of *A. moschatum* haplotypes are the same as in Fig. 2. The break in the line for *D. tenuipes* indicates that data were missing.

DNA sequence-based analyses and comparison with other species

The mean nucleotide diversity for all 155 samples of *A. moschatum* was 0·00021, which is 4·8 and 3·5 times lower than that observed in the co-occurring species, *Nothofagus cunninghamii* and *Tasmannia lanceolata* respectively (Table 1). Nucleotide diversities differed between individual fragments for each species (Table 1). Indel diversity per site was also lower in *A. moschatum* (0·00012) than in either *N. cunninghamii* (0·00019) or *T. lanceolata* (0·00031).

The distributions of pairwise mismatches between cpDNA haplotypes were strongly multimodal in *N. cunninghamii* and *T. lanceolata* at the species level and when analysed for different regions (Fig. 4). In contrast, for *A. moschatum*, the mismatch distributions were unimodal, apart from the mainland where the most diverged haplotypes in *A. moschatum* were observed in the most northern part of the species' mainland range.

Comparative mutation rates

Rates of SSMY were between 1·79 and 3·34 × 10^{-4} for the branch leading to *A. moschatum* when estimated using the calibration with *Laurelia sempervirens*, while they were between 1·64 and 5·68 × 10^{-4} when using *Nemauron veillardii* for calibration (see Supplementary Data Table S5). Apart from

TABLE 1. *The nucleotide diversity* ($\pi \times 10^{-3}$) *estimated for* Nothofagus cunninghamii *(213 samples),* Tasmannia lanceolata *(244 samples) and* Atherosperma moschatum *(155 samples) for each fragment and the whole alignments*

	N. cunninghamii	*T. lanceolata*	*A. moschatum*
*trn*S-*trn*f_M	**2·16 ± 0·14**	–	–
*rps*16 intron	**0·05 ± 0·04**	–	–
*trn*L-*trn*F	**1·66 ± 0·26**	0·35 ± 0·11	*
*psb*M-*trn*D	1·09 ± 0·18	**2·14 ± 0·21**	(0·00 ± 0·00)
petN-*psb*M	0·14 ± 0·05	**0·27 ± 0·08**	0·23 ± 0·05
K1-*mat*K1	–	**0·44 ± 0·07**	0·40 ± 0·07
*mat*K6-K2	–	**0·38 ± 0·05**	0·24 ± 0·05
*trn*L intron	–	**0·18 ± 0·05**	0·00 ± 0·00
trnT-*trnL*	–	–	0·08 ± 0·05
*atp*B-*rbc*L	–	–	(0·00 ± 0·00)
Whole alignment	1·01 ± 0·1	0·73 ± 0·06	0·21 ± 0·03

The highest values for each fragment are indicated in bold.
A dash denotes fragments that were not studied for the species and an asterisk denotes a fragment that did not amplify.
Nucleotide diversities in parenthesis were calculated with only 24 samples.

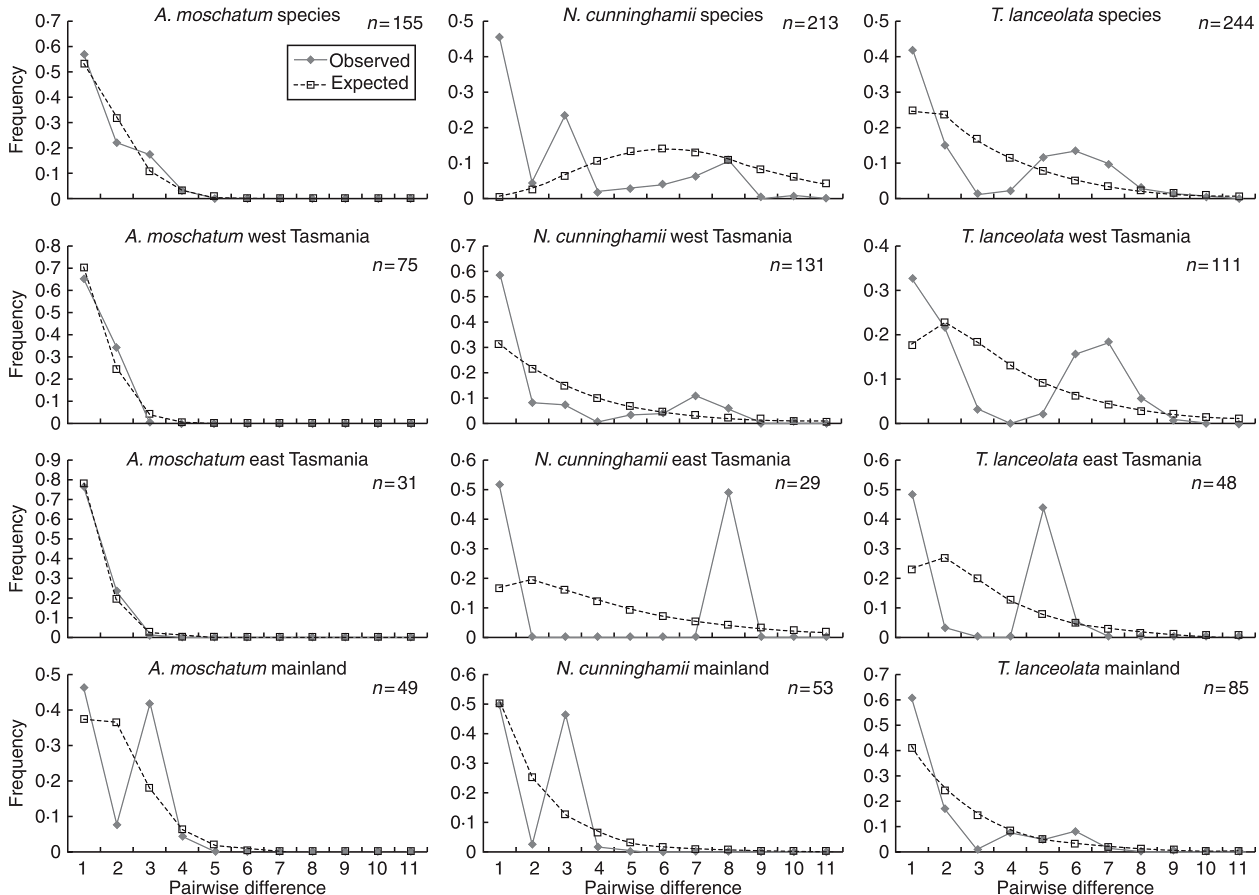

FIG. 4. Distributions of pairwise mismatches between cpDNA haplotypes for the species (*Atherosperma moschatum*, *Nothofagus cunninghamii* and *Tasmannia lanceolata*) and populations in three regions (western Tasmania, eastern Tasmania and south-east mainland Australia). Expected frequencies of pairwise differences in a growth/decline population model are indicated by broken lines. Observed frequencies of pairwise differences between haplotypes are indicated by continuous lines. Note the marked bi- or trimodality in all regions for both *N. cunninghamii* and *T. lanceolata*, but mostly unimodal distributions for *A. moschatum*.

the higher rates when using the youngest divergence times of *A. moschatum* and outgroup species, these values were similar to the cpDNA substitution rate of *rbcL* inferred for the family Atherospermataceae of $1{\cdot}4–2{\cdot}4 \times 10^{-4}$ SSMY by Renner *et al.* (2000).

DISCUSSION

Low chloroplast variation

The chloroplast variation observed in *A. moschatum* appears to be unusually low, with lower estimated nucleotide diversity than 27 of 28 other woody plant species for which values were available (Fig. 5). The only lower estimate was based on a comparatively smaller sample size in a rare and mostly insular tree species, *Laurus azorica* (Rodriguez-Sanchez *et al.*, 2009). Although direct comparisons of cpDNA diversity with other plant species can be affected by the choice of cpDNA fragments and the intensity and coverage of sampling, the value for *A. moschatum* is so low that, in conjunction with the widespread sampling of its range, it appears unlikely to be due to such effects. We argue that the paucity of variation is likely to be due to a reduction of genetic diversity in the species (i.e. via bottlenecks) rather than a recent origin of the species or low rates of molecular evolution.

This low genetic diversity is unlikely to be the result of recent evolution or arrival in south-eastern Australia. This is because the genus is monotypic and endemic to the region, and molecular dating based on a phylogeny encompassing all genera in the family and the sequencing of 4203 bp of the chloroplast genome provides an age of 69·5 million years for the genus (Renner, 2004). Furthermore, there is macrofossil evidence (a leaf fossil that is indistinguishable from leaves of the extant species) for the presence of the species in western Tasmania in the Early Pleistocene, approx. 1 million years ago (Hill and Macphail, 1985).

The possibility that the low diversity of *A. moschatum* can be explained by low cpDNA substitution rates can be discounted for this species. It is plausible that rates of molecular evolution may have been relatively low in *A. moschatum* because it has the ability to regenerate asexually from basal sprouts, possibly extending the lifespan of individuals (Hickey *et al.*, 1982; Read and Hill, 1988*b*), a trait that can be associated with low rates of mutation in angiosperms (Smith and Donoghue, 2008). However, the cpDNA substitution rates estimated for *A. moschatum* (between

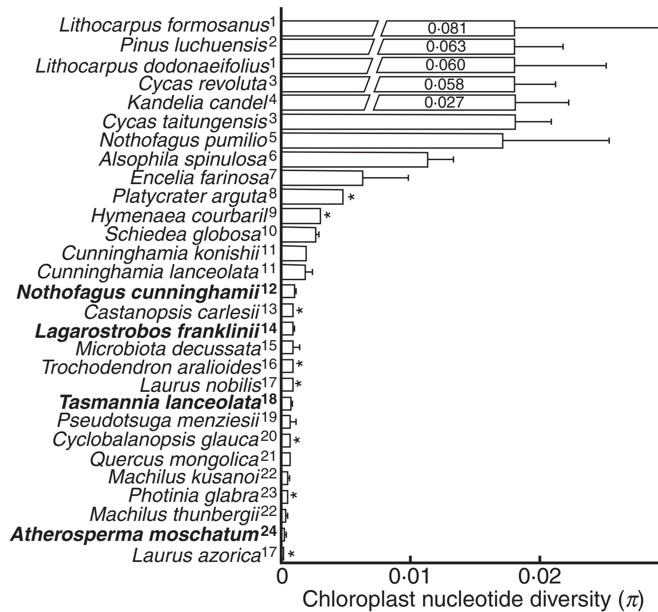

FIG. 5. Chloroplast nucleotide diversity (π; Nei, 1987) ± s.e. for 29 woody plant species, including *Atherosperma moschatum*. All Australian cool temperate rainforest species are shown in bold. High values have been truncated with actual values shown on the bar. No standard errors were available for species indicated with an asterisk. Only values calculated from species with more than ten samples were included. Sources: 1, Chiang *et al.* (2004); 2, Chiang *et al.* (2006); 3, Chiang *et al.* (2009); 4, Chiang *et al.* (2001); 5, Mathiasen and Premoli (2009); 6, Su *et al.* (2005); 7, Fehlberg and Ranker (2009); 8, Qiu *et al.* (2009); 9, Ramos *et al.* (2009); 10, Wallace *et al.* (2009); 11, Hwang *et al.* (2003); 12, Worth *et al.* (2009); 13, Cheng *et al.* (2005); 14, Clark and Carbone (2008); 15, Artyukova *et al.* (2009); 16, Huang *et al.* (2004); 17, Rodriguez-Sanchez *et al.* (2009); 18, Worth *et al.* (2010); 19, Gugger *et al.* (2010); 20, Huang *et al.* (2002); 21, Okaura *et al.* (2007); 22, Wu *et al.* (2006); 23, Aoki *et al.* (2006); 24, this study. Full details are given in Supplementary Data Table S6 (available online).

$1{\cdot}64–5{\cdot}68 \times 10^{-4}$ SSMY) were similar or higher in comparison to values estimated for *T. lanceolata* ($1{\cdot}28–1{\cdot}51 \times 10^{-4}$ SSMY) and *N. cunninghamii* ($1{\cdot}23–2{\cdot}6 \times 10^{-4}$ SSMY) in this study and by Albert *et al.* (1994) for both Winteraceae ($0{\cdot}98$ to $1{\cdot}47 \times 10^{-4}$ SSMY) and Nothofagaceae ($2{\cdot}10 \times 10^{-4}$ SSMY). Thus, although there is considerable uncertainty in substitution rate estimates (Renner *et al.*, 2000; Kay *et al.*, 2006) there is no reason to suppose that *A. moschatum* has slower molecular evolution of the chloroplast than co-occurring species with higher levels of chloroplast diversity.

One possible explanation for the low chloroplast diversity of *A. moschatum* would be major reductions in population size (i.e. bottlenecks) in the past due to cold intolerance. This hypothesis is consistent with marked ecological and physiological differences between *A. moschatum* and the more genetically diverse species *N. cunninghamii* and *T. lanceolata*. Unlike *A. moschatum* which rarely occurs near or above the tree line (Hill *et al.*, 1988), both *N. cunninghamii* and *T. lanceolata* form large populations well above the tree-line (Macphail, 1975). Experimental evidence suggests that *A. moschatum* is the least cold tolerant of the tree species in cool temperate rainforest (Read and Hill, 1989; Read and Busby, 1990; Feild and Brodribb, 2001). For example, after a single freeze–thaw cycle, *A. moschatum* was shown to be the most vulnerable of five other Tasmanian rainforest woody species to loss of hydraulic conductivity (Feild and Brodribb, 2001). In addition, *A. moschatum* had the lowest frost resistance and lowest photosynthetic tolerance to low temperatures of all other Australian cool temperate trees studied (Read and Hill, 1989; Read and Busby, 1990).

During the LGM, treeless conditions are thought to have extended as far north as Barrington Tops (Sweller and Martin, 2001). Stands of tall forest, like those in the present day, would have been highly restricted in the LGM due to lower rainfall and a depressed tree-line, with the tree-line being near current sea-level in Tasmania (Colhoun, 1985). Based on evidence of the extent of glaciation, Early Pleistocene glacials may have been colder than the LGM (Kiernan, 1990; Colhoun *et al.*, 1996). Thus, the habitat for *A. moschatum* during glacials may have been considerably more restricted than those of *N. cunninghamii* and *T. lanceolata* resulting in very small populations of *A. moschatum*, thereby reducing existing variation by genetic drift (i.e. the bottleneck effect). Moreover, such bottlenecks may have occurred repeatedly over the Pleistocene glacial/interglacial cycles.

Repeated bottlenecks, dispersal and multiple refugia

The star-like chloroplast network observed in *A. moschatum* implies that the species has undergone a major bottleneck, or the cumulative effect of multiple bottlenecks, that reduced the chloroplast diversity within the species to a single haplotype in the past. An alternative explanation is that this major loss of diversity in *A. moschatum* may have involved a selective sweep, although this is difficult to detect with chloroplast data alone. This haplotype was most likely to have been haplotype H1 or an extinct ancestor, the zero branch length leading to haplotype H1 on the phylogram (Fig. 3) suggesting that haplotype H1 is plesiomorphic, retaining ancestral states to all other haplotypes observed in *A. moschatum*. Any major bottleneck and subsequent recovery leading to the occupation of its current widespread range are unlikely to have occurred in the recent past (i.e. since the LGM) as this scenario would require rapid evolution of five haplotypes during range expansion, two of which are characterized by multiple differences from the ancestral hapotype H1. In fact, the modern geographic structuring of haplotypes is likely to have developed over multiple glacial periods involving establishment of new populations via rare dispersal events and glacial survival and bottlenecks (i.e. refugia) within multiple regions. The clearest evidence for the effect of glacial refugia comes from Victoria and NSW, where three regions contain a single, endemic-derived haplotype. Apart from the possibility of selection, this chloroplast divergence is best explained by bottlenecking events subsequent to the major event described above, because it involves either the *in situ* loss of ancestral haplotypes or dispersal of the derived haplotype to the new region and extinction of that same haplotype in its ancestral area.

On the mainland the chloroplast evidence supports survival within at least one glacial refugia in each of three regions: near Monga in south-eastern NSW; the Blue Mountains/Barrington Tops region NSW; and within the current range of haplotype

H3 in Victoria/far south-eastern NSW. The chloroplast evidence for the location of glacial refugia for *A. moschatum* in Tasmania is less clear. Haplotype H4 provides evidence for survival through the last glacial in at least one area in Tasmania, probably in western Tasmania where LGM fossil pollen of the species, albeit at very low levels, has been found (Macphail and Colhoun, 1985; Colhoun *et al.*, 1999; Hopf *et al.*, 2000). In addition, it is possible that the Tasman Peninsula, where all three Tasmanian haplotypes were found, represents an additional LGM refugia, an assertion that is supported by genetic evidence for two wet forest *Eucalyptus* species (Bloomfield *et al.*, 2011; Nevill *et al.*, 2010). However, the chloroplast pattern in this region could equally be explained by Holocene admixture, particularly given the absence of any fossil pollen record from this region. However, the sharing of single haplotypes across large geographic areas, particularly for H1 in Tasmania, H3 across the whole species range in Victoria and south-eastern NSW and H6 on both sides of the Hunter Valley, a dryland barrier for wet forest species situated between the Blue Mountains and Barrington Tops regions, limits the ability to locate precisely glacial refugia for the species. This is in contrast to *N. cunninghamii* and, in particular, *T. lanceolata* where high haplotype diversity and endemicity allowed comparatively fine-scale resolution of the location of refugia (Worth *et al.*, 2009, 2010).

Some hypothesis can also be made of the dispersal of the species. On mainland south-eastern Australia where populations are generally small and geographically isolated, all populations sampled contained a single haplotype. This contrasts with previous chloroplast phylogeographic studies of some trees with plumose wind-dispersed seeds. For example, admixture of haplotypes was observed across the range (98 samples) of *Populus tremula* in Italy (Salvini *et al.*, 2001). A similar finding was observed in *Salix caprea* across Europe (Palme *et al.*, 2003). Despite having a similar dispersal mechanism, *A. moschatum* may differ from these aforementioned tree species due to its non-pioneer status and generally poor recruitment (Hickey *et al.*, 1982; Read, 1985; Neyland, 1991), resulting in the relatively rare successful establishment of viable populations in either new habitat or already occupied areas. However, the constraints on dispersal may be much lower than in the co-occurring species, *Nothofagus cunninghamii* and *Tasmannia lanceolata*, which show strong, relatively small-scale geographic patterning of chloroplast haplotypes (Worth *et al.*, 2009, 2010). In mainland populations of *A. moschatum*, two additional factors are likely to have contributed to the lack of admixture of haplotypes – the fact that there are only three observed haplotypes in this region, and that mainland populations of the species are in most cases geographically separated by extensive inhospitable territory (>150 km in each case). Admixture of haplotypes may explain the co-occurrence of haplotypes in Tasmania, where the rare haplotype H2 occurs on both sides of the dry Midlands and all three Tasmanian haplotypes were found on the Tasman Peninsula. This discrepancy could be explained by a greater level of long-distance dispersal related to the greater propagule pressure of the species in Tasmania where the species is more common than on the mainland (Shapcott, 1994).

While it is plausible that the current distribution of *A. moschatum* has been impacted by extensive Holocene expansion within some regions, e.g. within Victoria, better understanding of the timing of any dispersal events across areas of unsuitable habitat is difficult and is likely to require the analysis of the nuclear markers such as nuclear microsatellites or sequence data. Nuclear markers may be particularly informative due to the fact that *A. moschatum* is insect pollinated and therefore likely to have restricted pollen dispersal (Shapcott, 1995). In addition, next generation sequencing technology could be used to simultaneously scan whole chloroplast genomes of multiple individuals (Parks *et al.*, 2009) to investigate whether widespread haplotypes are in fact differentiated in other parts of the chloroplast genome not investigated in this study.

Conclusions

Although *A. moschatum* is the most widespread of all cool temperate rainforest woody plants in Australia, the species chloroplast diversity was the lowest of any cool temperate rainforest plant studied to date and among the lowest documented among woody plants. Similar estimated rates of chloroplast substitution to *N. cunninghamii* and *T. lanceolata*, species that both have higher chloroplast diversity, mismatch analysis and geographic patterning of haplotypes provide evidence that *A. moschatum* underwent successive bottlenecks reducing existing chloroplast variation during the Pleistocene. A severe bottleneck several glacials ago may have reduced chloroplast diversity to a single haplotype. Further bottlenecks occurred during subsequent glacials, including the LGM, resulting in the restriction of endemic haplotypes to four separate regions across its range, south-western Tasmania, Victoria/far south-eastern NSW, Monga, and the most northern populations in NSW. Dispersal to new areas of suitable habitat plausibly played a role in the Holocene within these regions; however, detailed information on the history of populations within these regions awaits further investigation.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: Fossil pollen record of *Atherosperma moschatum* (Atherospermataceae) since 150 thousand years ago. Table S1: Sample collection information and haplotype for all 155 samples of *A. moschatum*. Table S2: Primer pairs tested in *Atherosperma moschatum* for screening for chloroplast variation. Table S3: Single nucleotide polymorphisms and SSR polymorphisms defining the six chloroplast DNA haplotypes observed in *A. moschatum*, shown in comparison to the most frequent haplotype. Table S4: The aligned length obtained for cpDNA fragments sequenced in *A. moschatum* and their GenBank accession numbers, and Genbank accession numbers for outgroups. Table S5: Jukes–Cantor corrected sequence divergences, divergence time and estimated mutation rates calibrated using alternative estimates of divergence ages between *A. moschatum*, *Tasmannia lanceolata* and *Nothofagus cunninghamii*, and outgroup taxa. Table S6: Chloroplast nucleotide diversity (π) for 29 plant species, including *A. moschatum*.

ACKNOWLEDGEMENTS

We thank John and Noela Cross, Jon Marsden-Smedley, Peter Woodard and Dave Woods for help with sample collection; Ken Gillanders for access to his private collection; and the Department of Sustainability and Environment (Victoria), the Department of Primary Industries, Parks, Water and Environment (Tasmania) and the Department of Environment, Climate Change and Water (NSW) for collection permits. We also thank two anonymous reviewers for their comments which helped to improve this manuscript. This work was supported by a Discovery grant DP0557260 from the Australian Research Council awarded to René Vaillancourt.

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